Dissertação de Mestrado

Toxicologia e Contaminação Ambientais

Study of anti-inflammatory bioactivity of cyanobacterial strains using murine macrophage RAW 264.7 cells

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“Even discredited and ignored by all, I can not give up, because to me, winning is never give up.”

Albert Einstein

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Abstract

Cyanobacteria are organisms capable of photosynthesis, and have an important role in the ecosystems, since they are involved in nitrogen and carbon cycles. They produce secondary metabolites of high diversity including toxins and bioactive compounds. Until present, novel compounds were discovered with anti-cancer, antiviral, antifungal, antibacterial and antiparasitic potential. The Blue Biotechnology and Ecotoxicology (BBE) group at CIIMAR, offers a unique collection of cyanobacteria isolated from water samples and solid materials from the Portuguese coast and have conducted studies regarding to the bioactive potential of marine cyanobacteria isolated from the Portuguese coast. Recent results revealed interesting bioactivities such as cytotoxicity in cancer cell lines, bacteria and fungi and toxicity in marine invertebrates.

In order to respond to the emergent need of natural anti-inflammatory compounds without toxic side effects, we performed the present work with the aim to evaluate for the first time the anti-inflammatory potential of 13 cyanobacteria strains from the BBE cyanobacteria culture collection. The bioactivity of the cyanobacterial fractions was tested in *in vitro* cultures of the mice macrophage RAW 264.7 cell line. The evaluation of anti-inflammatory activity was executed by the Griess method.

The strains *Phormidium* sp LEGE 06363 and *Aphanizomenon* sp. LEGE 03283 exerted the strongest effects, and were selected for further sub-fractionation using wet chemistry extraction techniques as solid phase extraction (SPE) and high pressure liquid chromatography (HPLC). After a process of gravity chromatography, the fraction E14026 A7 of the strain *Phormidium* sp LEGE 06363 showed anti-inflammatory potential in RAW 264.7 cells. Unfortunately, the complete isolation of the compound was not possible, since the biomass of the subfraction was not enough. The fractions E14035 A2J7, A2J9, A2J10 and A2J13 from the strain LEGE 03283 demonstrated anti-inflammatory effects, and NMR's analysis was performed to verify the complexity of the compounds. These subfractions still contain more than one compound. Consequently, further purification is needed in order to isolate the responsible compound for the bioactivity.

This study represents a first step in the study of the bioactive anti-inflammatory potential of cyanobacteria from the BBE collection. Strains with interesting potential were identified, but additional methodologies must be performed to finally purify and isolate the responsible compound and to decipher their main molecular targets.

Resumo

As cianobactérias são organismos conhecidos por serem capazes de realizar o processo fotossintético e possuem um papel importante nos ecossistemas, uma vez que estão envolvidas nos ciclos do azoto e carbono. São também responsáveis por produzir diversos metabolitos secundários, que adquiriram importância com a recente pesquisa do potencial de bioatividade. Atualmente, estudos científicos já permitiram a descoberta de novos compostos com forte capacidade anticancerígena, antiviral, antifúngica, antibacteriana e antiparasitário. O grupo BBE no CIIMAR, oferece uma coleção única de cianobactérias isoladas de amostras de água e de materiais sólidos a partir da costa Portuguesa e foram realizados estudos sobre o potencial bioativo de cianobactérias marinhas isoladas do litoral Português. Resultados recentes revelaram bioatividades interessantes, tais como citotoxicidade em linhas celulares de cancro, bactérias e fungos e toxicidade em invertebrados marinhos.

A fim de responder à necessidade emergente de compostos anti-inflamatórios naturais sem ou com menos efeitos secundários que os AINEs, realizou-se o presente trabalho com o objetivo de avaliar o potencial anti-inflamatório de 13 espécies de cianobactérias, da coleção de culturas de cianobactérias do grupo BBE. A bioatividade das frações das cianobactérias foi testada em culturas *in vitro* da linha celular de macrófagos de ratos RAW 264.7. A avaliação da atividade anti-inflamatória foi executada através do método de Griess.

As estirpes *Phormidium* sp LEGE 06363 e *Aphanizomenon* sp. LEGE 03283 foram as que exerceram efeitos mais fortes e selecionadas para posterior sub-fracionamento utilizando técnicas de química de extração como a extração em fase sólida (SPE) e cromatografia líquida de alta pressão (HPLC). Depois de um processo de cromatografia, por gravidade, a fração E14026 A7 da estirpe *Phormidium* sp LEGE 06363 mostrou potencial anti-inflamatório na linha celular RAW 264.7, embora o isolamento do composto não foi possível, uma vez que o peso da fração não era o suficiente. Depois de um período de purificação, as frações E14035 A2J7, A2J9, A2J10 e A2J13 de LEGE 03283 demonstraram efeitos anti-inflamatórios, e foi realizada a análise de NMR para verificar a complexidade dos compostos. As frações são ainda complexas, por isso, é preciso um outro período de fracionamento e purificação de modo a isolar o composto.

Longe de este estudo estar completo, representa apenas um primeiro passo no estudo do potencial anti-inflamatório bioativo de cianobactérias marinhas. Outras metodologias adicionais devem ser realizadas para realmente purificar e isolar o composto que é responsável por exercer este efeito e para confirmar os resultados.

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List of abbreviations

APC - antigen presenting cells

MHC - major histocompatibility complex

TL - T lymphocyte

NK – Natural Killer

ROS - reactive oxygen species

H_2O – Water molecule

O_2 – Oxygen molecule

OH – Hidroxyl

H_2O_2 – Hydrogen peroxide molecule

O_2^- - Radical superoxide

NO - Nitric oxide

nNOS –neuronal nitric oxide synthase

iNOS – inducible nitric oxide synthase

eNOS - endothelial nitric oxide synthase

CaM – calmodulin

NF-IL-6/IL-6 - nuclear factor interleukin-6

(STAT)-1α - signal transducer and activator of transcription

IRF-1 - interferon regulatory factor-1

TGF-β - growth factor β

LPS - lipopolysaccharide

NO^{2-} - nitrite

NO^{3-} - nitrate

NSAID - non-steroidal anti-inflammatory drugs

IMS - Technology company and health information

USA – United States of America

COX – cyclooxygenase

AIDS – HIV Virus

GI - gastro intestinalis bleeding

FDA – Food and Drug Administration

TNF-α – tumor necrosis factor

PS - polysaccharides

PGE_2 – prostangladin E_2

MCP 1 - monocyte chemoattractant protein-1

IL-4 - nuclear factor interleukin-4

IL-5 - nuclear factor interleukin-5

IL-10 - nuclear factor interleukin-10

MP - macrophage cells

CNS - central nervous system

PM - Primary microglia

DMEM – Dulbecco's Modified Eagle Medium

FBS – Fetal Bovine Serum

DMSO – Dimethyl Sulphoxide

KDO - 3-deoxy-D-mannooctulosonic Acid

SULF – Sulfanilamide

NEDD – N-(1-naphtyl) ethylerediamine dihydrochoride

H3PO4 – Phosporic acid

NaNO2 – Sodium nitrite

NMMA – N-Monomethyl-L-arginine

$NaNO_2$ -Sodium nitrite

UV – Ultra-Violet

HPLC – High Performance Liquid Chromatography

EtOAc – Ethyl Acetate

SPE – Solid Phase Extraction

NMR - Nuclear magnetic resonance

qRT-PCR – Quantitative real time Polymerase Chain Reaction

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1. Introduction

1.1 Innate immune system VS adaptive immune system

The immune system is constituted by two major subdivisions: the innate or non-specific immune system and the adaptive or specific immune system. Both major subdivisions of the immune system possess cellular and humoral characteristics by which they carry out their protective task. Even though these two arms of the immune system have distinct functions, they interact between each other (i.e., components of the innate immune system influence the adaptive immune system and vice versa) (Cruvinel *et.al.*, 2010; Mayer, 2016).

Innate immunity represents the first line of defence of a mammalian organism in order to respond to a large, however limited number of stimuli. It is represented by physical, chemical, and biological barriers, expert cells and soluble molecules, is not specific for an antigen or immunogens, and, after contact, it remains the same in quality and quantity. This system has defences that are present and ready to be mobilized when an infection is realized. Consequently, in the first hours and days after the exposure to new stimuli, we trust on our innate immune system to protect us from infection (Mayer, 2016; Alberts *et.al.*, 2002; Cruvinel *et.al.*, 2010).

Adaptive immune system acts like a second line of defence and protects the organism against re-exposure to the same offending agent. However, it takes a while to respond for the first time when in contact with a new invading organism. Since its initiation depends on activation of specialized cells, as specific clones of lymphocytes B and T, this organisms' defence can take a week before the responses turn efficient. This process occurs because the adaptive immune system demonstrates immunological memory. It has the ability to "remember" the last time it has encountered an offending agent and so it reacts more quickly on subsequent exposure to the same organism (Mayer, 2016; Alberts *et.al.*, 2002; Cruvinel *et.al.*, 2010).

Acquired response is constituted by the main followed features: self-control, specificity and diversity of recognition, immunological memory, expert response and tolerance to components of the organism itself. The main cells involved in this kind of defence are lymphocytes, still antigen presenting cells (APC's) play a key role in its activation, offering antigens associated with molecules of the major histocompatibility complex (MHC) to T lymphocyte (TL) (Mayer, 2016; Alberts *et.al.*, 2002; Cruvinel *et.al.*, 2010).

Component/Features	Innate immunity	Adaptive immunity
Main differences	Antigen-independent response	Antigen-dependent response
	Not antigen-specific	Antigen-specific
	Instantaneous and maximal response after exposure	Long time between exposure and maximal response
	No immunologic memory	Immunologic memory
Cells	Phagocytes (dendritic cells, macrophages, and neutrophils) Natural killer (NK) Mast cells, eosinophils and basophils	NK/T, B, and T lymphocytes Dendritic cells or APC's
Soluble molecules	Complement Acute phase proteins Cytokines Chemokines	Antibodies Cytokines Chemokines

Table 1: Main differences between innate immune system and adaptive immune system (adapted from Cruvinel *et. al.*, 2010 Mayer, 2016)

1.2 Concept of inflammation

Several diseases and health problems are related to the inflammation of tissues. An inflammatory response is a symptom of a lesion or tissue infection recognized by an increased vascular permeability and plasma extravasation. This process brings cells and molecules, like mediators, leucocytes and an increase of fluid, from the host's defences to the inflammation sites where they are needed to destroy the infective agent (Oliveira *et.al.*, 2014; Kumar *et.al.*, 2015; Damte *et.al.*, 2011; Heras *et.al.*, 2009)**.** Generally, inflammation suggests a harmful reaction, but it is considered a protective response that is essential for human and animal survival. It could have several causes like infections, trauma, tissue necrosis, immune responses and foreign bodies (Kumar *et.al.*, 2015). Generally, acute and chronic inflammation is distinguished.

Chronic vs acute inflammation

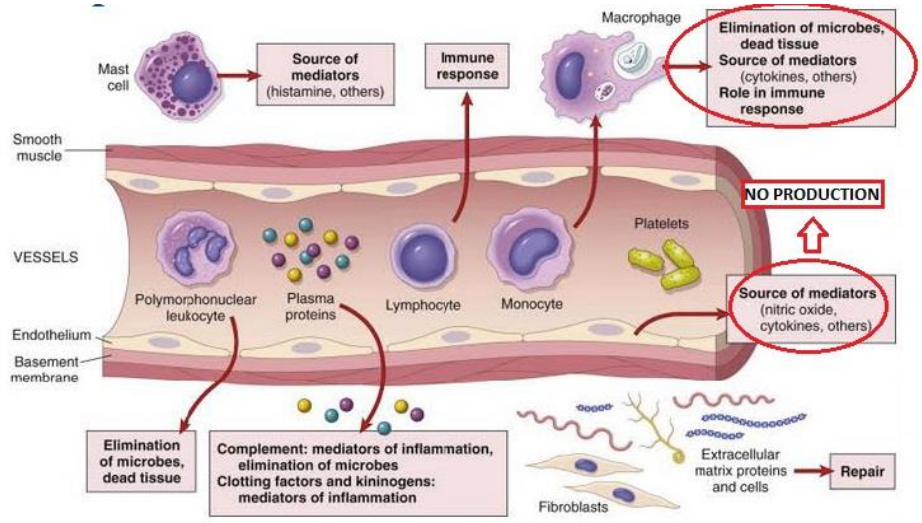


Figure 1: Mechanisms of acute and chronic inflammation and their principal mediators (adapted from Kumar *et.al.*, 2015)

Acute Inflammation

Every time a host's body meets an injurious agent, for example, an infectious microbe or a dead cell, components of the cells such as phagocytes try to eliminate these agents, as a first defence of the organism (innate immune system). Their goal is to eradicate these infectious agents. Acute inflammation is a short-term process that involves a vascular response and the recruitment of polymorphonuclear granulocytes, typically neutrophils, followed by monocytes, that later, *in situ*, can differentiate into macrophages.

Major components of Acute Inflammation
Increase in blood flow due to vasodilatation
Increase permeability, leading to leukocytes and plasma proteins to leave the circulation
Movement and accumulation of leukocytes at the site of infection and their activation

Table 2: Major components of acute inflammation (Kumar *et.al.*, 2015)

First, phagocytes and other defence cells are responsible to release some mediators of the inflammation process, like cytokines, prostanglandines, kinins, lipid messengers, etc. This kind of mediators can act on small vessels in the adjacent area, encouraging the efflux of plasma and the emigration of leukocytes to the site of injury. Second, the leukocytes are activated by the infectious agent and the mediators. Their activation allows the elimination of the infectious agent. During this process, the infectious agent is

destroyed and the host can be healthy again. In the case of the foreign compound do not be fast eliminated, it can develop to a chronic inflammation.

Some acute inflammation symptoms are redness, warmth and swelling as a response to the increased blood flow and edema. The leukocytes present in the blood flow adhere, traverse the endothelium and travel to the site of injury, activated by chemotactic agents. Once they get activated, they are able to release proteases and toxic metabolites to the outside of the cell, which produces tissue damage. Pain is a result of such damage and the release of prostaglandins, neuropeptides, and cytokines (Kumar *et.al.*, 2015; Heras *et.al.*, 2009; Jo *et.al.*, 2010).

Chronic Inflammation

Chronic inflammation is a process that can follow acute inflammation or may begin insidiously. It is characterized by a reaction of prolonged duration, in which inflammation, tissue injury and efforts of repairing coexist, in numerous combinations.

Chronic inflammation can be derived from different causes, such as:

a) Persistent infections by microorganisms difficult to eradicate (mycobacteria, virus, fungi).

b) Hypersensitivity diseases**,** like auto-immune diseases that occur when auto-antigens evoke a self-perpetuating immune reaction, which results in chronic tissue damage and inflammation. This is based in an excessive and inappropriate activation of the immune system (asthma, multiple sclerosis, rheumatoid arthritis).

c) Continued exposure to toxic agents, either exogenous or endogenous; this type of inflammation, leads to an *in situ* activation of numerous cell types such as macrophages, plasma cells, lymphocytes, other leukocytes and the respective formation of mediators. The dominant cells are macrophages, which have an important role, since they contribute to the reaction by secreting mediators such as cytokines and growth factors. These mediators behave as intermediates because they act on diverse cells, or activate other, especially T lymphocytes, which are able to destroy foreign invaders. The inflammatory reaction can be longer or bigger depending on the occurrence of a bidirectional interaction between these cells (Kumar *et.al.*, 2015; Jo *et.al.*, 2010).

Feature	Acute	Chronic
Onset	Fast (minutes/hours)	Slow (days)
Cellular infiltrate	Neutrophils	Monocytes, macrophages, lymphocytes
Tissue injury, fibrosis	Commonly mild and self-limited	Often severe and progressive
Local and systemic signs	Prominent	Less prominent, can be subtle

Table 3: Features of acute and chronic inflammation (adapted from Kumar *et.al.*, 2015)

Generally, an inflammatory response by an organism involves the release of reactive oxygen species (ROS) through activated neutrophils and macrophages (Oliveira *et.al.*, 2014).

Reactive Oxygen Species

Reactive Oxygen Species (ROS) is the term that includes several oxygen radical compounds and some non-oxidizing radical compounds such as hypochlorous acid, hydrogen peroxide, ozone, etc. ROS are compounds, which have a tendency to donate oxygen atoms to other substances. Typically, highly reactive ROS possess more volatility and one or more unpaired electrons (Kumar, 2011; Ferreira *et.al.*, 1997). All biological systems produce ROS (Ferreira *et.al.*, 1997), which can derive from internal or external sources, such as: natural metabolic processes or exposure to radiation, air pollutants, industrial chemicals, respectively (Kumar, 2011).

In normal physiological conditions, O_2 is reduced, since it accepts four electrons, forming the H_2O molecule. Throughout this process, some ROS are formed, such as the superoxide radical (O_2-), hydroperoxyl (HO_2.), hydroxyl (OH) and hydrogen peroxide (H_2O_2). In a normal situation, the complete reduction of O_2 occurs within the mitochondria and the high reactivity of ROS is neutralized with the input of the four electrons (Ferreira *et.al.*, 1997).

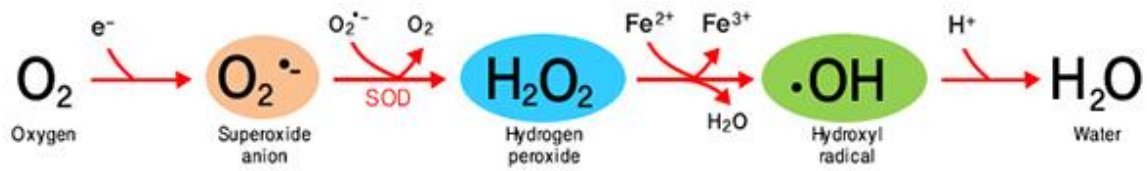


Figure 2: ROS formation (www.biospectrum.com)

In contrast, oxidative stress occurs when the balance is disturbed between the production and destruction of ROS. There must be equilibrium between the reduction and oxidation of these compounds, which is determined by the presence of electrons responsible for the redox flow pairs. Certain changes in a biological system that influence this balance can lead to oxidative stress. The intensity and danger of this processes depends essentially on the concentrations of antioxidants and pro-oxidants, the constant reaction rate with target molecules and cellular compartmentalization of these processes, which solubility and diffusion are essential (Vasconcelos *et.al.*, 2007).

Cases with inflammation of cells, as noted previously, may lead to excessive production of ROS and hence the triggering of oxidative stress process.

Nitric Oxide Production

Nitric oxide (NO) is a product resulting from the activation of macrophages by cytokines, microbial compounds or both. NO is derived from the oxidation of amino acid L-arginine by a family of isoenzymes, known as nitric oxide synthases (NOS): nNOS –neuronal nitric oxide synthase, iNOS – inducible nitric oxide synthase and eNOS - endothelial nitric oxide synthase and functions as a tumoricidal and antimicrobial molecule *in vitro* and *in vivo* (Bogdan, 2001; Speranza *et.al.*,2010; Johann *et.al.*, 2007). NO concept has been accepted as explained above, however in the last years the scientific community recognized NO essential in the immune system.

The production of NO is a characteristic of original immune-system cells (NK cells, dendritic cells, mast cells and phagocytic cells counting macrophages, Kupffer cells, neutrophils, monocytes, microglia and eosinophils), but it is certain that other cells are involved in immune feedbacks (endothelial cells, vascular smooth muscle cells, epithelial cells, keratinocytes, fibroblasts, chondrocytes, mesangial cells, hepatocytes, and Schwann cells) (Bogdan, 2001).

While cytokines are responsible for the regulation of the expression of iNOS, nNOS and eNOS, the activity of some proteins is switched on by the elevation of intracellular concentrations of Ca^{2+} and the binding of the protein calmodulin (CaM), as a response to neurotransmitters or vasoactive substances. Even at fundamental Ca^{2+} levels, this protein is strongly connected to iNOS and is distinguished from the constitutive isoforms due its prolonged production of a relatively large amount of NO (Bogdan, 2001; Musial & Eissa, 2001).

The three isoforms of NOS's may operate during immune responses in different levels of regulation (Bodgan, 2001). Cytokines regulate iNOS by activation of the iNOS gene

promoter, which has been analyzed systematically in epithelial cell lines, mouse macrophages, and human hepatocytes. In this process, diverse transcription factors are involved like NF-κB, AP-1, nuclear factor interleukin-6 (NF-IL-6), the signal transducer and activator of transcription (STAT)-1α, interferon regulatory factor-1 (IRF-1), and the high-mobility group-I(Y) proteins. The signalling pathway involved will be different depending on the cytokine or microbial stimulus, and the response to that will promote or inhibit iNOS expression. Therefore, there will be different feedbacks depending on the amount of NO produced: (i) low concentrations of NO activate NF-κB and make a positive feedback thus upregulating iNOS; (ii) high concentrations of NO have the opposite effect, which may help prevent NO overproduction (Bodgan, 2001).

One of the several mechanisms that suppresses the NO production in macrophages, by transforming the Growth Factor β (TGF-β) is the increased degradation of iNOS protein. The protein degradation is also responsible to control nNOS and iNOS involving the proteasome pathway. When in presence of lipopolysaccharide (LPS), the iNOS gene, in the macrophages, is stimulated and so, increase the amount of steady-state iNOS protein (Musial & Eissa, 2001; Johann *et.al.*, 2007).

NO is a compound that mediates many physiological functions, as a result, it is the main target to investigate in many different pathological conditions. However, NO, *per se*, has a problem regarding its quantification, since it has a short half-life (milliseconds or less, depending on the environment) when in the surrounded environment exists O_2 and other scavenging compounds, for example, haemoglobin (Giustarini *et.al.*, 2008). Consequently, many assays measure the presence of NO in an indirect way. Usually, some markers are preferred as an index of NO production. On one hand, those that accumulate from the stable degradation of NO, nitrite (NO^{2-}) and nitrate (NO^{3-}), S-nitrosothiols and on the other hand, those, which produce an increase in cGMP levels, due to NO dependent initiation of guanylyl cyclase (Tsikas, 2005; Giustarini *et.al.*, 2008). The final products of the NO oxidation pathways are nitrite and nitrate and, as a result, their concentration in human body fluids depends on NO production itself.

Lipopolysaccharides

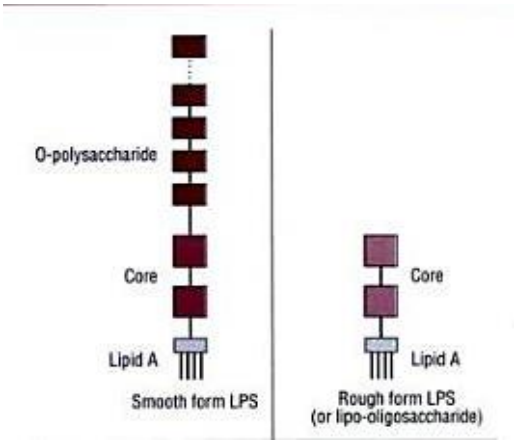


Figure 3: LPS composition (adapted from Parija, 2012)

Lipopolysaccharides (LPS) are normally used in bioassays to assess the inflammation process. An exposure of at least 4h to LPS induces an inflammation on the cells. The macrophages are then activated and start fighting against the LPS presence (Kellog *et.al.*, 2015; Dey *et.al.*, 2005; Johann *et.al.*, 2007). LPS are complex molecules, also known as lipoglycans and endotoxins, present in the external membrane of gram negative bacteria (Parija, 2012). Due to their structure and composition, LPS are considered an important toxin that protects the cell's membrane against certain chemical attacks, increases the negative charge of the cell's membrane and helps to stabilize the overall membrane structure.

It is an essential molecule to gram-negative bacteria, since its removal or mutation results in bacterial death (Raetz & Whitfield, 2002). Structurally, LPS are divided in three main parts: Lipid A, O antigen (or O polysaccharide) and Core oligosaccharide.

(1) Lipid A: Consists in a phosphorylated glucosamine disaccharide attached to a long chain of fatty acids. This hydrophobic fatty acid chain anchors the LPS into the bacteria's membrane, and the rest of the LPS projects from the cell surface. Lipid A is the LPS domain responsible for the toxicity of the LPS when released into the animal's cells. When the immune system attacks the bacterial cells, some fragments of membrane and Lipid A are released producing symptoms like fever, diarrhea and septic shock. Variations in lipid A structure among different species of gram-negative bacteria are short, remaining similar within the same species.

(2) O antigen: Has different sugars in its composition, depending of the strain. It is attached to the core extending outwards, to the surface of the bacterial cell, and as a consequence, is the target for recognition by the host antibodies.

(3) Core oligosaccharide: Includes two characteristic sugars – KDO and a heptose that are attached to the Lipid A. The LPS cores of many bacteria contain non-carbohydrate components, such as phosphate, amino acids, and ethanolamine substitutes (Parija, 2012).

1.3 Anti-inflammatory compounds from synthetic source

On the one hand, clinicians rely on drugs with the ability of soothe inflammatory processes as a consequence of an injury or diseases like rheumatoid arthritis, asthma, atherosclerosis, Alzheimer's, among others, which may be considered as recurrent excessive inflammation. On the other hand, anti-inflammatory drugs are usually used to treat moderate pain such as menstrual pain, headache and muscle pain. The most commonly used pharmaceuticals as treatment for inflammation can be non-steroidal and steroidal (Oliveira *et.al*, 2014; Alolga *et.al.*, 2015).

Non-steroidal anti-inflammatory compounds (NSAID's) are consumed by humans for the treatment of pain, fever and acute or chronic inflammation (Alolga *et.al*, 2015; www.CRBestBuyDrugs.org - Consumer Reports Best Buy Drugs). According to IMS Health (Technology company and health information), NSAID's are the most prescribed drugs in the USA with more than 98 million prescriptions in 2012 (www.CRBestBuyDrugs.org - Consumer Reports Best Buy Drugs). These drugs are compounds with different chemical molecules and structures and distinct therapeutic potential drugs, which have three features in common: (i) basic pharmacological properties, (ii) identical basic mechanism of action, as well as (iii) similar adverse effects. In addition, all drugs of this group have an acidic character. Most NSAID's are weak acids with pKa values ranging from 3.0 to 5.0 (medium strength acid) (Gouda *et.al.*, 2013).

These types of compounds are known for containing in its structure hydrophilic (carboxylic or phenolic group) and lipophilic groups (aromatic ring, halogen atoms). In the stomach, NSAID's acquire the lipophilic form, since they have acidic characteristics, whereas more propitious conditions in the small intestine favour the absorption of weak acids. In the plasma, these compounds are found in ionized form. They have a high affinity to plasma proteins (> 97%) and therefore aren't readily distributed in the extra-vascular systems. Their metabolism occurs mainly in the liver by oxidation processes and combination of inactive metabolites. These are subsequently excreted in the urine or bile (Gouda *et.al.*, 2013).

NSAID's are responsible for blocking the production of substances, which are called prostaglandins. These chemicals are derived from fatty acid metabolism via the route

COX (cyclooxygenase) and play a role in pain, inflammation, fever, asthma, glaucoma, osteoporosis, cancer, cardiovascular disease, birth and male sexual dysfunction (www.CRBestBuyDrugs.org - Consumer Reports Best Buy Drugs; Rao & Knaus, 2008). In low doses, NSAID's work essentially as pain relievers. In higher doses, these drugs reduce the inflammatory response due to tissue damage and relieve the pain associated with this inflammation. More specifically, they work by blocking the action of the enzymes COX-1 and COX-2, which are involved in the production of prostaglandins. For this, NSAID's mode of action is due to the inhibition of the prostaglandin production by inactivating enzymes involved in the process.

Although the treatment of inflammation is effective with the use of NSAIDs, the risks are often higher than the benefits. The prostaglandins produced by COX-1 enzyme may assist in the protection of the gastric mucosa and its absence leads to the risk of developing severe haemorrhages and ulcers. Moreover, the continued use of these drugs can still bring many side effects such as the injury of the gastrointestinal and renal systems, fluid and salts retention, heart attacks, strokes, and others (Qandil *et.al.*, 2012; Gouda *et.al.*, 2013; Rao & Kaus, 2008).

The NSAID's are divided into the following groups, regarding to their chemical structure: derivatives of salicylic acid (such as salicylamide, aspirine, sodium salicylate); aniline and derivatives of p-aminophenol (acetaminophen); pyrazolone derivatives (phenylbutazone, propyfenazona); oxicams (such as piroxicam, tenoxicam, meloxicam, between others); derivatives of the arylalkanoic acids (aceclofenac, diclofenac, indomethacin, between others); derivatives of the 2-arylpropionic acids (profens) (such as ibuprofen, flurbiprofen, naproxen, ketoprofen, tiaprofenic acid); N-arylanthranilic acids (fenamic acids) (like olfenamic acid, mefenamic acid flufenamic); enolic acid and coxibs derivatives (for example, rofecoxib, celecoxib, etoricoxib, etc.); naphtylbutanone derivatives (nabumetone); sulphonamide (nimesulide); derived benzoxazocine (nefopam).

Age	Risk of gastro intestinalis bleeding	Risk of death due to GI's bleeding
16-44	1 in 2100	1 in 12353
45-64	1 in 646	1 in 3800
65-74	1 in 570	1 in 3353
>75	1 in 110	1 in 647

Table 4: NSAID's risks (Adapted from www.CRBestBuyDrugs.org - Consumer Reporters Best Buy Drugs). GI, gastro intestinalis bleeding.

1.4 Pharmaceuticals from natural source

Despite the great advances with the synthetic drugs, natural compounds are still considered a suitable source for novel and efficient anti-inflammatory drugs, and a high percentage maybe used as novel compounds in clinical trials (Costa *et.al.*, 2014).

It is urgent to find other clinical techniques to treat diseases with symptomatic inflammation. Ideally, new and safer compounds will be developed without severe side effects as mentioned above. Medicinal plants, seaweed and some species of trees have been described as viable alternative sources of natural compounds with anti-inflammatory properties. Extracts of a species of tree growing in Mato Grosso, Brazil, *Dilodendron bipinnatum*, was shown to be capable of treating different situations of inflammation (Oliveira *et.al.*, 2014). Their mechanism of action involves the inhibition of the migration of cells and of mediators of inflammatory response, by inhibiting the Th1 and Th2 cytokine stimulation without harming the NO pathway (Oliveira *et.al.*, 2014). Other civilizations, such as Japan and China, often turn to brown algae for treatment of hyperthyroidism and other diseases associated with glands that are both examples for chronic inflammation diseases (Almeida *et.al.*, 2011).

In recent decades, the scientific community uncovered that algae and marine microalgae are one of the richest and most promising natural sources of primary and secondary metabolites. This type of organisms produces many compounds, such as carotenoids, terpenoids, xanthophyll, chlorophyll, vitamins, polyunsaturated and saturated fatty acids, amino acids, acetogenins, antioxidants, such as alkaloids, polyphenols, polysaccharides, halogenated compounds. Those compounds can have different bioactivities, e.g. antimicrobial, antifouling or UV light protection agents, cancer, AIDS, inflammation, pain, arthritis, or viral, bacterial or fungi infections (Almeida *et.al.*, 2011; Deig *et.al.*, 1974). Nowadays, algae are the source of about 9% of biomedical compounds obtained from the sea (Almeida *et.al.*, 2011; Jha *et.al.*, 2004). Natural products are still an important source for drug discovery, either used directly or as template for structural alterations. In 2010, approximately 10% of the drugs on the market were unaltered natural products, 29% derived from natural products and employed as template for novel compounds, and the remaining (61%) were synthetic drugs (Castro *et.al.*, 2016).

1.5 Cyanobacteria

Phytoplankton or microalgae are photosynthetic organisms that live suspended in the water and even though they represent less than 1% of Earth’s biomass, they are primary producers and very important since they are in the base of the food web. These

organisms can be divided in three main groups: diatoms, dinoflagellates and cyanobacteria (Hallegraeff, 2003; Leão *et.al.*, 2011).

Cyanobacteria are fascinating organisms derived from an ancestral strain of bacteria that can be found in different habitats and different varieties. They have a rather important role in the ecosystem, since they are involved in carbon and nitrogen cycles. (Morais *et.al.*, 2015; Dittman *et.al.*, 2015). Cyanobacteria have fossil records from over 2 billion years ago and they appear in various aquatic environments, from both salted water to fresh water, can have different sizes, colours, and shapes. They are the only bacteria capable of oxygenic photosynthesis and their high adaptability to each ecosystem could be the reason why they are so prolific in secondary metabolites (Morais *et.al.*, 2015; Miranda *et.al.*, 2001).

Cyanobacteria are a Phylum that belongs to the Bacteria Domain. They have 2 to 40 μm in diameter, and have a complex cell wall constituted by a thick peptidoglycan layer and periplasmic space between the internal cytoplasmic membrane and the external cell membrane. The outer cell membrane consists of lipopolysaccharides, phospholipids and proteins, while the intracellular space is composed of proteins, enzymes and metabolites (Madigan *et.al.*, 2010).

Cyanobacteria taxa were determined according to the Botanical Code of Nomenclature and include five major groups (Rippka *et.al.*, 1979):

I (*Chroococcales*) — unicellular cyanobacteria that divide by binary fission or budding, II (*Pleurocapsales*) —unicellular cyanobacteria that reproduce by multiple fission, III (*Oscillatoriales*) — non- heterocystous filamentous cyanobacteria that split in only one plane, IV (*Nostocales*) — heterocystous and filamentous cyanobacteria that split in only one plane, V (Stigonematales) —filamentous heterocystous cyanobacteria that reproduce in more than one plane (Boone & Castenholz, 2001).

Bioactive Compounds from Cyanobacteria

Cyanobacteria are usually known for the formation of toxic blooms in various water systems around the world, contributing to the intoxication level of public health, due to the release of toxic compounds (Dittman *et.al.*, 2015). However, in the last years, investigation has been made to better understand the nature of certain metabolites produced by cyanobacteria, with the ultimate goal of finding new substances with applications for human health. Many of those cyanobacterial compounds have shown to have biological activity relevant to human health (Dittman *et.al.*, 2015). Today, several drugs have been developed and obtained from marine natural sources (Singh *et.al.*,

2011). 24% of the commercially available natural products for biomedical research are of cyanobacterial origin (Gerwick & Moore, 2012). The most famous example of a cyanobacterial compounds with biomedical application is Brentuximab vedotin, a synthetic compound with origin from a cyanobacterial metabolite, which passed the FDA approval for clinical use as anticancer drug (Katz *et.al.*, 2011).

These bioactive compounds may be obtained directly from the primary metabolism, such as proteins, fatty acids, vitamins and pigments or from the secondary metabolism (Dittman *et.al.*, 2015; Singh *et.al.*, 2011).

Since studies from Richard E. Moore in the 70's, evidence was presented that marine cyanobacteria could be an efficient source of secondary metabolites relevant to biological activities especially in the fight against cancer and neurotoxicity (Nunnery *et.al.*, 2010). Natural products obtained from cyanobacteria can be divided in a diverse range of structural classes, including peptides, polypeptides, alkaloids, lipids, and terpenes. Their structures can be complex with a range of unusual modification (Dittman *et.al.*, 2015). Such compounds may show a broad spectrum of activities, such as antifungal, antiviral, anti-enzyme, antibiotic, anti-tumor, anti-bacterial and protease inhibitors (Dittman *et.al.*, 2015; Singh *et.al.*, 2011). Up to now, more than 800 metabolites have been described from various taxa and beyond that, one single strain of cyanobacteria is able to produce a wide array of structurally different secondary metabolites. Filamentous marine cyanobacteria from subclass III (Oscillatoriales) represent nearly half of the 800 compounds reported so far (Gerwick *et.al.*, 2008).

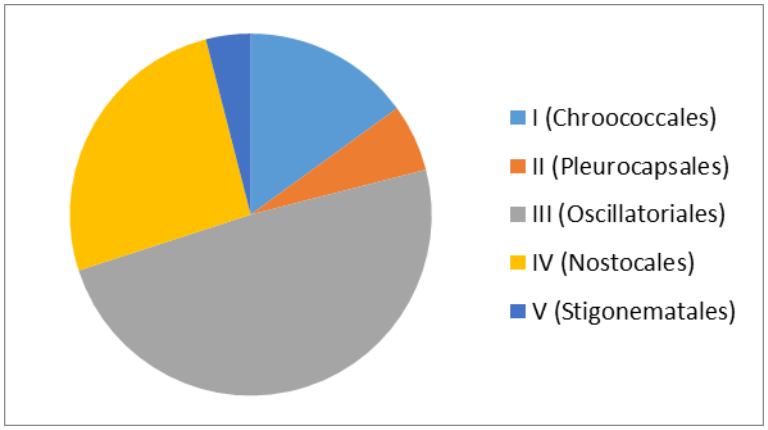


Figure 4: Distribution of reported cyanobacterial secondary metabolites through the five major botanical sub-classes (adapted from Gerwick *et.al.*, 2008).

The biosynthesis of mixed metabolites originates from an intermingling of genes responsible for encoding biosynthetic enzymes, which have the function of linking amino

acids and acetate subunits. In most cases, the metabolites resulting from the cyanobacteria activity are from a natural arrangement, since it is possible that they can appear as combinations of different biosynthetic subunits and under several juxtapositions (Nunnery *et.al.*, 2010).

During evolution, cyanobacteria revealed a bright ability to produce this kind of bioactive compounds, which could be related to their need to defend them against predation by other organisms (Dale *et.al.*, 1999). An emerging trend exists to find compounds with pharmacological capabilities from marine cyanobacteria, in contrast to the past perspective, where cyanobacteria were only considered as producer of a range of toxic metabolites. To illustrate this, recent studies revealed some natural cyanobacterial compounds with anti-inflammatory properties (Villa *et.al.*, 2010). This perspective has been revolutionizing the world of cyanobacteria and updates the knowledge we had about secondary metabolites from marine cyanobacteria.

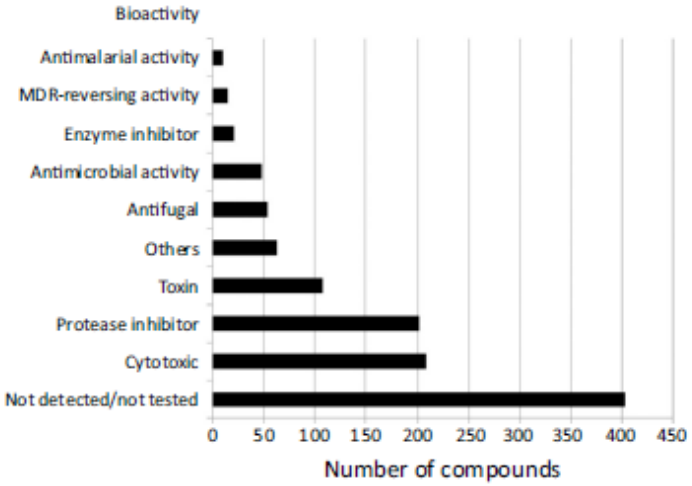


Figure 5: Number of compounds extract from cyanobacteria and their bioactivities (Adapted from Dittmann *et.al.*, 2015)

Anti-Inflammatory Activity

Many new compounds were discovered after extract fractions with real potential to treat diverse diseases, but the focus has, however, been in discovering new drugs for cancer treatment. Some of these new molecules were or are still in preclinical and/or clinical assays (Gerwick *et.al.*, 2008). In the following, we refer to studies dealing with anti-inflammatory bioactivity of plants, seaweed and cyanobacteria.

Plant' source

A recent study (Iwamoto *et.al.*, 2015) revealed that the species *Piper umbellatum*, known in Brazil as "malvarisco", is a promising source of natural compounds with anti-

inflammatory activity and anti-cancer, since it is bioactive both *in vitro* and *in vivo*, and has no side effects, even at high doses.

A last year study (Shaikh *et.al.*, 2015) revealed that some species of plants have anti-inflammatory activity, e.g. *Terminalia bellarica, Cissus quadrangularis, Plumbago zeylanica, Terminalia chebula.* The ethanolic extracts were shown to produce a significant decline in the volume of carrageenan induced mice paw edema after 1 hour (32.85% and 34.28%, respectively) by the oral administration of different doses, e.g. 250 mg/kg of *T. bellarica* and *T. chebulla.* Indomethacin (20 mg/kg) - standard anti-inflammatory medicine - was used as control to compare the results of this investigation, which showed effective inhibition (51.48 %) at 5 h. Both medicinal plant species *Terminalia* (*T. bellarica* and *T. chebula*) are the major constituents of a popular formulation called 'Triphala', prescribed by clinical physicians and most of the traditional healthcare practitioners in India and many Asian countries.

Mathew *et.al.* (2013) showed that ethanolic and aqueous extracts from *Kalanchoe Pinnata (Lam.) Pers (*Family-Crassulaceae) have anti-inflammatory activity. They were tested against carrageenan induced paw-edema in rats and compared to the standard drug - indomethacin. Both types of extracts had anti-inflammatory activity by reducing the volume of paw edema in rats at two different doses, 300 and 600 mg/kg.

Costus speciosus (Koen ex.Retz.) Sm. (crepe ginger, family Costaceae) is originate from Malay Peninsula of Southeast Asia and it is an ornamental plant. This plant is used for the treatment of various diseases in the Indian traditional medicine, such as: burning sensation on urination, fever, flu, flatulence, leprosy, helminthiasis, skin disorders, asthma, hiccough, rheumatism, bronchitis, inflammation and anaemia. The n-hexane–$CHCl_3$ soluble extract from *C. speciosus* rhizomes contained a new eudesmane acid, specioic acid (8) and more seven known compounds. Compounds 1–4 showed potent anti-inflammatory activity, followed by 7 and 8. These results support the use of this plant in traditional treatments of inflammatory conditions (Al-Attas *et.al.*, 2015).

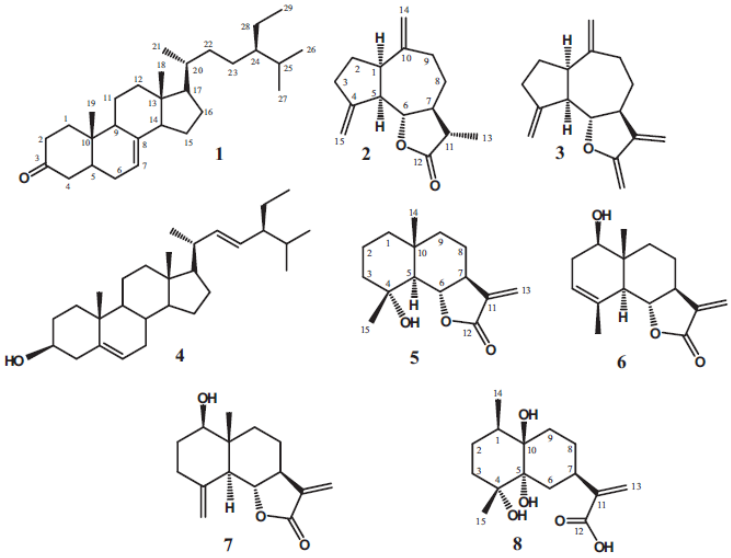


Figure 6: Chemical structures of the compounds isolated (1-8) from the extract of *C. speciosus* (Adapted from Al-Attas *et.al.*, 2015)

The phytochemicals present in Asian individual spices and herbs already revealed to suppress proinflammatory mediator expression. Regarding to Red curry paste, that is a Thai dish composed by five to seven herbs and spices, the results were astounding. In RAW 264.7 macrophage line, phytochemicals present in the red curry paste ethanolic extract significantly decreased the NO's production through the inhibition of the expressions of COX-2, TNF-α, iNOS and IL-6 in a dose-dependent manner. This study was able to isolate and identify four flavonoids and three carotenoids that contribute for the antioxidant and anti-inflammatory activity (Tuntipopipat *et.al.*, 2011).

Seaweed source

Seaweed were shown to be a source of natural compounds with anti-inflammatory activities. Seaweed derived polysaccharides (PS) are the most widely studied group of metabolites that are the main part of seaweed walls, and may be involved in recognition mechanisms between pathogens and seaweeds. The sulphation of PS leads to a diversity of biological properties such as anti-viral, anti-inflammatory, anticoagulant, and anti-tumoral activities (Jiao *et.al.*, 2011). The most studied sulphated PS are fucans, which are made of a fucose backbone. Fucoidan is the best well known fucan and it was primarily isolated from brown algae by Kylin in 1913. These fucose-containing sulphated PS principally consist of a backbone of (1 → 3)- and (1 → 4)-linked *α* -L-fucopyranose residues, that may be organized in strectches of (1 → 3)- *α* -fucan or of alternating *α* (1 → 3) and *α* (1 → 4)-bonded L-fucopyranose residues. The residues of L-fucopyranose can be replaced by sulphate on C-2 or C-4. Fucoidan has been examined with carefully in the

last years since it was recognized as having a role in the biology of seaweed and for its bioactivity in several organisms (Morya *et.al.*, 2011; Li *et.al.*, 2008; Fitton, 2011). Another study demonstrated that fucoidan was responsible to inhibit strongly the excessive production of PGE_2 and NO in LPS-stimulated BV2 microglia (Park *et.al.*, 2011). This compound is capable of attenuate the expression of enzymes involved in the inflammation process such as monocyte chemoattractant protein-1 (MCP 1), iNOS and COX-2 and pro-inflammatory cytokines, as IL-1β and TNF-α. MCP-1 is a pro-inflammatory chemokine that promotes monocyte recruitment into an inflammatory site; once activated, the recruited cells may produce more pro-inflammatory mediators, inducing the inflammation process (Park *et.al.*, 2011). Fucoidans are still unexploited as a source of medicines due to their heterogenicity and plant source, regardless of their notable bioactivity and no reported oral toxicity. There are several experimental studies using *Fucus vesiculosus*, a vulgar harvest crop of a northern hemisphere kelp, as a source of fucoidan (Souza *et.al.*, 2006).

Sacran is another example of anti-inflammatory sulphated PS derived from marine algae, and was extracted from *Aphanothece sacrum* (an edible blue-green microalga that grows in a river in the Kyushu region, Japan). Sacran exerts anti-inflammatory effect on 2,4,6-trinitrochorobenzene-induced allergic dermatitis in mice, *in vivo*, once it improves the skin barrier function and diminish cytokine production (Ngatu *et.al.*, 2012). Sacran was responsible to diminished the scratching behaviour and inhibited the development of allergic dermatitis skin lesions. In addition, sacran efficiently inhibited IFN- *γ*, TNF-*α*, IL-4, IL-5 expression and the infiltration of eosinophiles in allergen-exposed ear skin.

A study performed *in vivo* and *in vitro* mice macrophage demonstrated that the extract from the marine alga *Gracilaria verrucosa* had an increasing effect on innate immunity by both oral and intraperitoneal administration. Does mean that compounds present in this alga can improve host defense activity against tumors and infection, and that can be beneficial for supplements in food with immunopotentiating activity for humans and domestic animals (Yoshizawa *et.al.*,1996).

In the last decades, in Europe, the consumption of algae has increased since 15 to 20 different species have been commercialized in France, Greece and Italy. In some western countries like Venezuela, USA and Canada, the macroalgae are used in industry as a source of hydrocolloids agar, carrageenan and alginate (Almeida *et.al.*, 2011).

Cyanobacteria source

Considering anti-inflammatory activity, few studies were published from cyanobacteria sources. However, a study from 1984 showed that marine life forms possessed anti-

inflammatory activity, such as anti-inflammatory compounds from the cyanobacterium *Rivularia* sp. (Baker, 1984). More recently, a study was carried out using the inhibition of NO on a mouse macrophage cell line (RAW 264.7) as target, in order to find new metabolites from marine cyanobacteria. In this study, the authors concluded that many malyngamides, especially of the F series, had a high inhibiting capacity, and thus, an anti-inflammatory activity. Various concentrations of malyngamide F acetate were analysed for its effect on NO production in RAW 264.7 cell line after LPS stimulation to determine its dose–response characteristics and potency. The assays revealed an IC_{50} of 3.4 µg/ml (7.1 µM) and showed that NO production was significantly inhibited in a concentration-dependent manner. Malyngamide F acetate is the compound of these secondary metabolites that did not reveal cytotoxicity, but had more efficient NO inhibitory activity. TNF-α was up-regulated by the treatment with malyngamide F acetate, almost 2fold compared to the LPS. TNF- α transcription increase, in contrast to the decrease in the cytokines IL-1β, IL-6, and IL-10 transcription, what suggests that malyngamide F acetate may be acting upon the MyD88-dependent inflammatory pathway, because usually LPS leads to a higher TNF-α transcription and decreases IL-1β in the MyD88 knockout mouse by means of the MyD88-independent pathway (TRIF-dependent). To find out more about the interactions between MyD88-dependent and -independent pathways, in this work, the authors, analysed the transcription of IL-10 and TNF-α in CpG and Poly I:C treated cells. Cytokines, included TNF-α were inhibited by the CpG activation of TLR9. Also, Poly I:C treatment, which activates TLR3 via a MyD88-independent pathway, with and without malyngamide F acetate application did not showed significantly differences in the transcription of IL-10 and TNF-α. That means malyngamide F acetate acts by selectively inhibate the MyD88-dependent pathway (Villa *et.al.*, 2010 (b)).

Silambarasan *et.al.* (2011) analyzed the anti-inflammatory potential of the aqueous extract of *Trichodesmium erythraeum* – a marine cyanobacterial species, found in tropical and subtropical areas. This study used albino wistar rats as model system *in vivo* and the inflammation was induced by the administration of carrageenan. After 30 minutes within the treatment with extracts from *Trichodesmium erythraeum,* the inhibition of paw edema in the rats was of 57.5 ± 5.5 % to the high dose (500 mg/Kg) and 47.5 ± 4.7% to the lowest dosage (300 mg/kg).

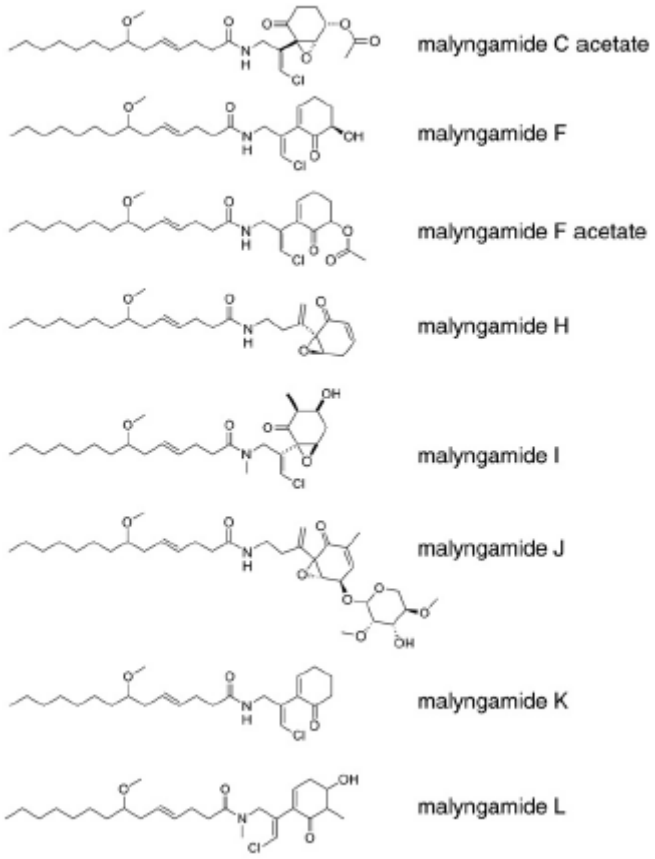


Figure 7: Different structures of the malyngamides extract from *Lyngbya majuscula*. These compounds are secondary metabolites which combine fatty acyl chains with polyketide synthase extended amino acid subunits (Adapted from Villa *et.al.*, 2010 (b))

1.6 Model Systems

In scientific investigation and molecular biology, cell culture has an important role as a tool. Cell culture is used to study toxicity assays, cancer research, virology, genetic engineering, gene therapy, cell-based manufacture, drug screening or development. In the last 20 years, macrophage cells (MP) entered into the focus of several laboratories, since they revealed a special role in immune regulation and tumor immunity. Macrophage cell lines can be obtained in many different ways: (i) from tumours or tumour-like cells; (ii) by culturing bone marrow cells using a factor agent that stimulate the macrophage colony- and continuing the continuously split of MP's; (iii) hybridomas are obtained by the fusion of diverse kinds of immortalized cells with normal macrophages. The use of MP lines offers many advantages: guarantee a homogeneous population and a large number of cells. It is also important to refer that the development of *in vitro* systems to replace animal use in science satisfies the controversy and criticism from animal rights groups (Chapes *et.al.*, 1988).

Macrophages

Today, many kinds of macrophage cell lines are available for scientific studies: (i) RAW 264.7 is a murine macrophage cell line; (ii) P388D1 is a derivative of P388 selected for the high production of IL-1. The cells showed a high reactivity in cell-mediated cytotoxicity antibody dependent. (iii) J774.A1 is derived from a tumor in a female BALB/c mouse that produces IL-5 and lysozyme. (iii) TPH1 is a human macrophage cell line (http://www.lgcstandards-atcc.org).

RAW 264.7 cell line (mouse monocyte macrophages) is a secondary cell culture derived from an induced tumour made by an intraperitoneal injection of Abselon Leukaemia Virus (A-MuLV) in a male mouse by and growing attached in a plastic flask or dish. Cells will pinocytose neutral red and phagocytose zymosan. This cell line is immortal, since they can divide several times from a “mother cell” (http://www.ecacc.org.uk). Stimulation of RAW 264.7 can be achieved through LPS endotoxin from gram negative bacteria. Once activated, MP’s play a critical role in inflammatory response and secrete nitrogen mediators and pro-inflammatory cytokines such as IL-1, IL-6 or TNF-α. This cell line is a good model system to study inflammatory processes and to evaluate the effects of adenosine receptor ligands as pro- or anti-inflammatory drugs.

Microglia cells

Microglia are innate immune cells of the brain and the local phagocytes. These cells have been recognized due to their broad role in the brain’s innate immunity, inflammatory disorders of central nervous system (CNS) and homeostasis (Henn *et.al.*, 2009, Stansley *et.al.*, 2012). The colonization of CNS by microglia takes place during embryonic development in rodents, at a prenatal stage (Stansley *et.al.*, 2012). These cells have a similar activation’s behaviour to macrophages and exhibit great functional plasticity when activated. They are a good model system to study inflammation diseases in the brain, like Alzheimer and Parkinson, and most frequently used are primary microglia and BV-2 or N9 cells (Henn *et.al.*, 2009, Stansley *et.al.*, 2012).

Primary microglia (PM) cells are used recurrently in scientific research in neuro-inflammatory studies, due to the similarities in phenotype to *in vivo* cells. These cells derived from the cortex of a rat or mouse before or early after birth (Stansley *et.al.*, 2012). The functional characteristics of these cells, like cell surface markers and secretory products are one of the advantages to use PM cells directly from the animal. These cells react really well to LPS-stimulation, by producing NO, prostaglandin E2 (PGE2), ROS, pro-inflammatory cytokines (TNF-α, IL-6 and IL-1) and MCP-1 (Stansley *et.al.*, 2012, Park

et.al., 2011). Their ability to proliferate is quite limited, so they need to be isolated freshly for each experiment and it is necessary 15-30 brains of rodents to produce microglia cells for a limited amount of experiments on signalling or disease mechanisms. In the biomedical research, significantly higher numbers are required, which has a huge impact on overall numbers of animal use. An alternative cell line could be better appropriate, once it would save time, animals and valued consumables, and facilitate research work. The process in this cell line is great to analyse cell markers and enzymes, however sometimes the time for preparation it is too much elevated, making this model less attractive than others (Henn *et.al.*, 2009, Stansley *et.al.*, 2012).

BV-2 cells come from raf/myc-immortalised murine neonatal microglia and are often used as a substitute for primary microglia. In the process of development of this line, microglia were purified by agitation, incubated overnight with control or J2 retrovirus containing supernatants in cell specific complete medium. They were incubated for about 3-4 weeks, and proliferating cells were observed in infected cultures, where non-infected cultures lost adherence and died (Stansley *et.al.*, 2012). BV-2, stimulated by bacterial surface molecules (LPS), can indeed trigger activation in astrocytes as assessed by translocation of the transcription factor NF-κB and secretion of the cytokine IL-6 (Stansley *et.al.*, 2012). Henn *et.al.* (2009) examined the BV-2 cells as an appropriate alternative to the primary cultures. In this study, when in presence of LPS, almost the same genes (90%) induced by the BV-2 cells were also induced by primary microglia; however, the up-regulation of genes in the BV-2 was far less pronounced than in primary microglia, leading to a less NO production comparing to PM, but still in considerable amounts. The BV-2 cell line allows technical viability between the procedures and an efficient preparation time (Stansley *et.al*., 2012). BV-2 cells are essential to inflammation studies, since microglia have an important role, not only in inflammation process, but also in toxicity conditions, especially in neurotoxicity.

Zebrafish

Another different method to evaluate the anti-inflammatory potential of an unknown compound is through the zebrafish (*Danio rerio*). This species is a tropical freshwater fish include in the family (Cyprinidae) of the order Cypriniformes. It is a popular fish for aquarium and it is native to the Himalayan region. Zebrafish are similar with mammals, including humans, since they share their functional homology and have analogous genetic sequence (Dahm, 2006). The reproduction is an advantage since it can generate a high number of offspring really quickly. Its embryos are robust, large, and translucent, and able

to develop in the outer environment (Spence *et.al.*, 2007; Dahm, 2006) However, zebrafish are not a collectively ideal research model. Associated to them exist some disadvantages to their scientific use, as for example the absence of a standard diet (Penglase *et.al.*, 2012) and the presence of some important differences relative to the role of some genes involved in human disorders.

Zebrafish larvae are quite small and are easy to manipulate and observe and also the ability of the compounds be pooled to the water bath and immediately absorbed when administered in a <1% DMSO solution, therefore, can be used as model system for inflammation studies. Wittmann *et.al.* (2012) proposed a new methodology to execute screening of small molecules with the aim of identifying immune modulatory compounds. The used larvae have a singular combination of presenting the property of transgenic lines expressing fluorescent proteins in leukocytes and the advantage of being optically clear. This combination allows the measurement *in vivo* of an acute inflammatory process (Wittmann *et.al.*, 2012).

Humanized mice

A humanized mouse is a mouse that it is able to carry functional human genes, cells, tissues, and/or organs. These mice usually are used as mammalian models, for human therapeutics in biological and medical research. Because they do not have host immunity, they are used as a recipient to human cells, since they can easily accept heterologous cells. The process begins when the scientist injects human hematopoietic stem cells (hu-CD34^{+}) on female mice. Twelve weeks after the procedure, the engraftment of mature human white blood cells (hCD45^{+}) is confirmed and considered successfully humanized. Humanized mice are a great model system, for long-term *in vivo* studies and to analysing the effectiveness and safety of potential novel drugs to modulate immune system (Brehm *et.al.*, 2014, Peltz, 2013; www.jax.org)

2. Objectives

Studies performed earlier at the Blue Biotechnology and Ecotoxicology Group (BBE) of CIIMAR had already established that some cyanobacterial strains are producers of cyanotoxins and secondary metabolites with interesting bioactivities towards human diseases (Martins *et.al.*, 2005). Anti-inflammatory activities of cyanobacterial strains from the cyanobacterial culture collection of CIIMAR (LEGE CC, http://www.ciimar.up.pt/legecc/) were not studied yet.

Therefore, the work presented in this thesis has as main objective to analyse the potential of cyanobacterial strains to produce anti-inflammatory compounds, which could be used in the future for the treatment of inflammatory diseases. The following specific objectives were defined:

- Growth of cyanobacterial biomass
- Production of extracts and fractions from different species of cyanobacteria
- Setup of assay conditions for the screening of anti-inflammatory activity
- Test extracts and fractions *in vitro* for their anti-inflammatory bioactivity
- Follow a bioassay-guided isolation process to purify a novel compound of cyanobacterial origin with anti-inflammatory activity.

3. Material and Methods

3.1 Culture of cyanobacteria strains

The Blue Biotechnology and Ecotoxicology Group of CIIMAR has a large collection of cyanobacterial strains, isolated from water samples and solid materials from the Portuguese coast and freshwater ecosystems (LEGE CC, http://www.ciimar.up.pt/legecc/). Culturing of cyanobacteria strains was performed in order to obtain a high quantity of total biomass. Cyanobacteria were grown in Z8 medium (25 g/L) (Kotai, 1972) and maintained at constant temperature of 25ºC with a light intensity of approximately 30 µmol photons m^{-2} s^{-1} and a light/dark cycle of 14:10h.

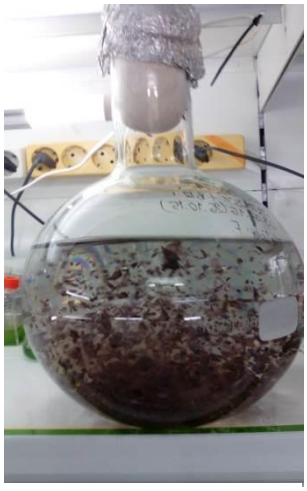


Figure 8: 6L flask LEGE 06144 strain

Figure 9: 10L flask LEGE 06148 strain

Figure 10: 10L flask LEGE 11427 strain

3.2 Collection of the biomass

The collection of biomass of the 3 different strains LEGE 06144, LEGE 06148 and LEGE 11427 was executed using the methods of centrifugation or filtration depending of the type of strain. During this work biomass of those strains was collected, but not used in these analyses due to the long phase of growth to have sufficient biomass for bioassay-guided fractionation and structure elucidation. So, in this work the strains used i*n vitro* assays were cultured and extracted in a previous research project (MARBIOTECH).

Also, the collected cyanobacterial biomass (MARBIOTECH) followed the standard procedure of the BBE group in CIIMAR, and so it was submitted to a normal phase vacuum liquid chromatography which resulted in 9 - 11 fractions

3.3 Cell culture of RAW 264.7

The murine RAW 264.7 macrophage cell line was used to study the anti-inflammatory bioactivity of cyanobacterial compounds *in vitro* (European Collection of Authenticated

Cell Cultures (ECACC), supplied by Sigma-Aldrich). Cells were cultured in DMEM Glutamax medium (Dulbecco's modified Eagle Medium GlutaMAX™) from Life Technologies (Thermo Fisher Scientific, Waltham, Massachusetts, USA), supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco-Invitrogen), 1% (v/v) of penicillin-streptomycin (Pen-Strep 1000 IU mL^{-1}) (Life Technologies) and 0.1 % (v/v) of amphotericin B (250 μg/mL) (Life Technologies). Cells were maintained in a humidified atmosphere with 5% of CO_2 at 37ºC in an incubator 8000 DH (Thermo Scientific, Waltham, MA, United States of America). Cells were cultured in petri dishes (Orange Scientific), and three times a week (Monday, Wednesday, Friday) the culture was passaged and split into new Petri dishes. The detachment of the cells was executed only with a micropipette, through a process of "up and down" until all the cells were detached, since cells have semi-adherent properties. Cells were centrifuged at 1600 rpm for 10 minutes to form a pellet. Cell density was calculated using the Countess™ Automated Cell Counter (Thermo Fisher Scientific) with 10 µL of medium of suspended cells mixed with 10 µL of trypan blue. Cell density for seeding was $1.3x10^5$ cells/cm^2. From time to time, some aliquots were frozen at -80˚C in medium supplemented with 10% of FBS and 5% of DMSO.

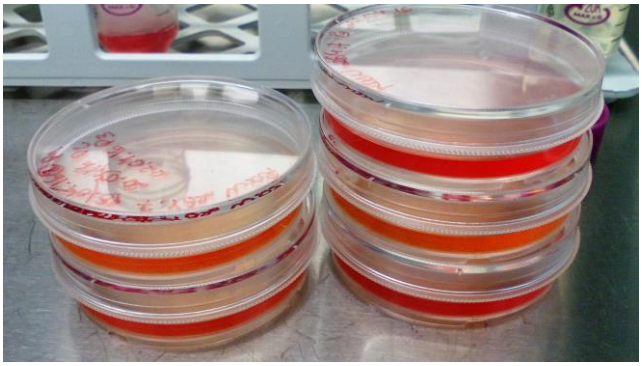


Figure 11: RAW 264.7 cell line cultured in petri dishes with complete DMEM (Picture of 25.04.2016, P3)

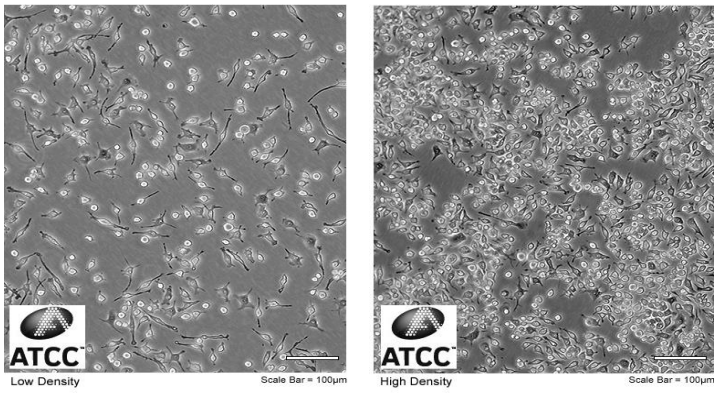


Figure 12: RAW 264.7 macrophage cell line under the microscope

3.3.1 Griess reaction for anti-inflammatory bioactivity

Once the immune system is activated, the production of nitric oxide - NO - occurs massively in cells, such as macrophages and mast cells. For this reason, the levels of NO are used as markers to detect inflammation by the Griess reaction, a simple and commonly applied technique (Miranda *et.al.*, 2001).

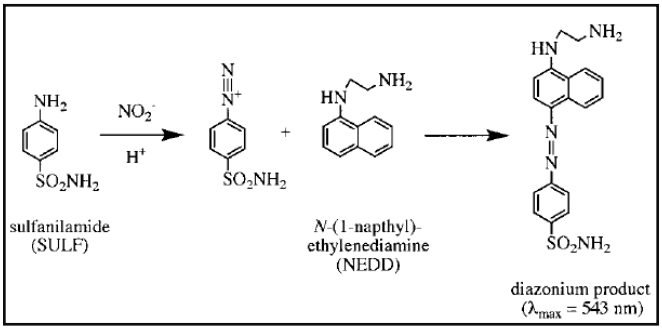


Fig 13: NO production during the cell inflammation (adapted from Miranda *et.al.*, 2001)

Reagents:

- N- (1- naphthyl) ethylenediamine dihydrochloride (NEDD)
- Sulfanilamide (SULF)
- Sodium nitrite
- Phosphoric acid (H_3PO_4)
- Ultra-pure water
- NG -monomethyl -L-arginine
- Lipopolysaccharide (LPS)

Preparation of Griess solutions:

a) SULF solution was prepared using ultra-pure H2O at 1% (w/v), or 0.1 g / 10 ml. For more effective dissolution, the solution was warmed in the water bath at 35ºC and sonicated by ultrasounds.

b) A solution of 5 % H_3PO4 was prepared from 85% stock solution by adding 1 ml to 16 ml of H_2O ultrapure.

c) NEDD solution was prepared at 0.1 % (w/v), with 0.01 g / 10 ml solution in 5% H_3PO4.

Note: Each solution must be filtered to remove particles.

Preparation of standard solution:

a) Sodium nitrite at 1 mM was used as standard in the assay. The solution was prepared by dissolving 0.0069 g in incomplete DMEM medium followed by magnetic stirring. This solution must be prepared freshly and can be stored in aliquots at -20° C.

b) In a 96-well microplate, a dilution series of 1: 2 is made using 1 mM sodium nitrite diluted in incomplete DMEM. Complete DMEM medium (as in cell culture) is used as a control in triplicates.

c) The 1st well is filled with 200 µL of sodium nitrite solution. All the remaining wells are filled with 100 µL of incomplete DMEM. Afterward, 100 µL of the 1st well are removed and placed into the second well, making "up and down" about 5 times before refilling the tip and moving to the next well. The same procedure is repeated to the following wells until no more wells are available.

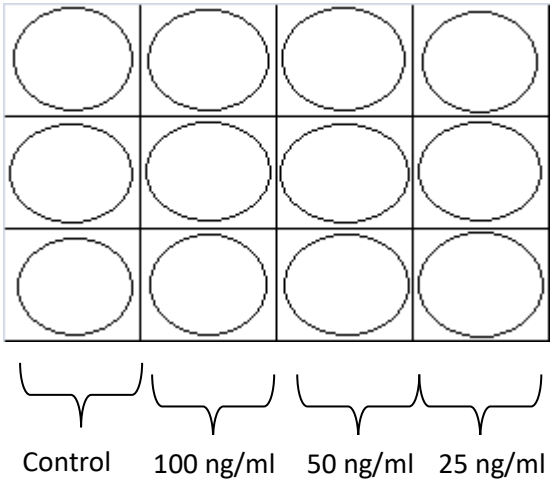
d) The last 100 µL are discarded along with the tip.

e) Finally 100 µL of the Griess solutions have to be added and the plates incubated in the dark for 10 minutes.

g) The plate is analyzed by spectrophotometry at 540 nm.

Induction of inflammation in RAW 264.7 cells:

a) This procedure is done in a 24 well microplate seeded with 500 µL RAW 264.7 cells at a density of $1x10^5$ cells/cm^2. Cells were allowed to attach and grow for 24h. In this example, four treatment groups are seeded in triplicates (see Figure below).



Preparation of stock solution of LPS (L4391 - 1mg – SIGMA; *Escherichia coli* 0111:B4) to induce inflammation:

a) Dilute 1 mg of LPS in 1 ml of incomplete DMEM (concentration of stock 1 mg/ml) and store in aliquots of 10 µLin Eppendorfs at -20 ° C.

b) The stock is diluted 1:1000 dilution in order to get a working solution with a final concentration of 1 µg/ml. Each time, 14 µL of the stock solution were diluted in 14 ml of

complete DMEM and vortexed for about 1 minute. Then 500 µl was transferred into each well of a 24-well microplate, where cells were seeded 24 hours before.

c) After 24 hours of incubation the exposure is carried out with the Griess test solutions. In order to do so, 100 µL of the medium in this plate are removed and placed in 96-well microplate;

d) 100 µL of Griess mixture is added to each well in a ratio of 1: 1 and allowed to incubate for 10 minutes in the dark;

e) After the incubation the samples are read in the spectrophotometer at 540 nm;

Preparation of NMMA solution (M7033-5MG - SIGMA):

a) NMMA will serve as a positive control in assays, since it is an inhibitor of NO production;

b) Prepare a NMMA solution at 10 mM concentration diluting 0.000248 g NMMA in 10 ml H_2O or incomplete DMEM.

d) NMMA solution is diluted 100x to get a concentration of 100 µM.

e) This solution is added during the treatment with LPS (final concentration of 1 µM in the medium) to verify that actually reduces NO levels, as expected.

f) The remaining solution should be stored in tightly sealed aliquots at -20 ° C for 1 month without degrading. If used again, it should be allowed to warm up at room temperature for 1 hour.

3.4 Screening of different extracts

In this work, 13 distinct strains of cyanobacteria from the BEE collection (Fig. 14-17) were recovered and 117 different fractions tested for their bioactivity. Fractions were tested in RAW 264.7 cells for testing anti-inflammatory activity. The Table 5 gives an overview about the tested strains. After the incubation of the cells for 24 hours, LPS was used at a concentration of 1 µg/ml to induce inflammation, followed by the exposure to the extracts and respective fractions. The fractions were prepared in DMSO with a final concentration of 10 mg/ml. In each well of a 24-well microplate, 1.5 µl were added of each fraction (final concentration of 30 µg/mL). The solvent control contained 0.3% DMSO. The Figure 18 shows an example of an exposure in a 24-well microplate.

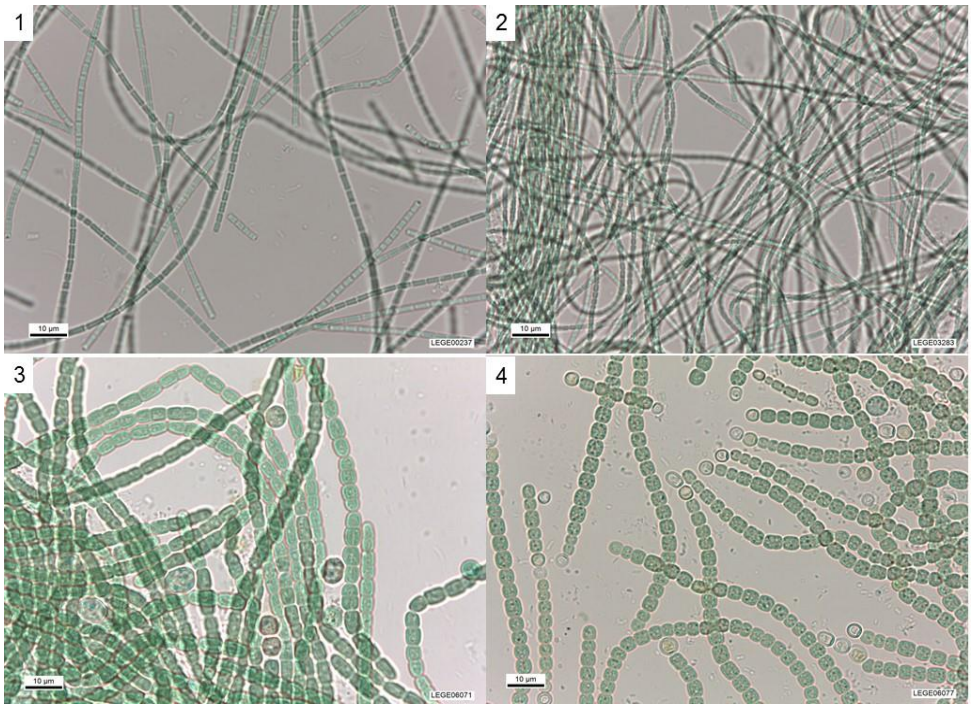


Figure 14: Microscopy's view of cyanobacteria strains. 1: LEGE 00237; 2: LEGE 03283; 3: LEGE 06071; 4: LEGE 06077 (From BBE collection)

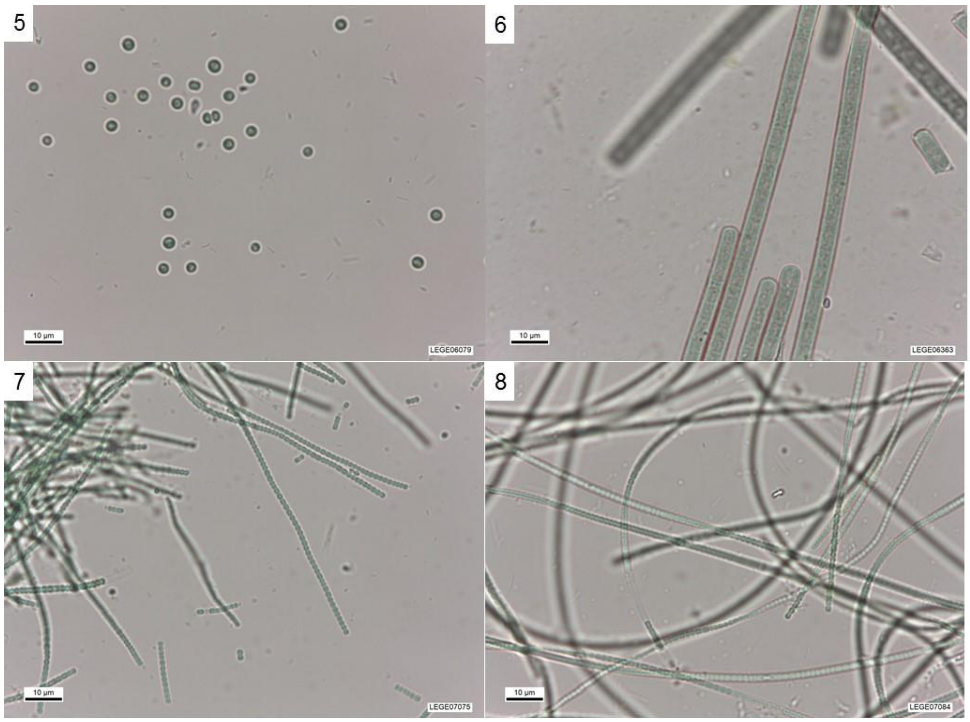


Figure 15: Microscopy's view of cyanobacteria strains. 5: LEGE 0679; 6: LEGE 06363; 7: LEGE 07075; 8: LEGE 07084 (From BBE collection)

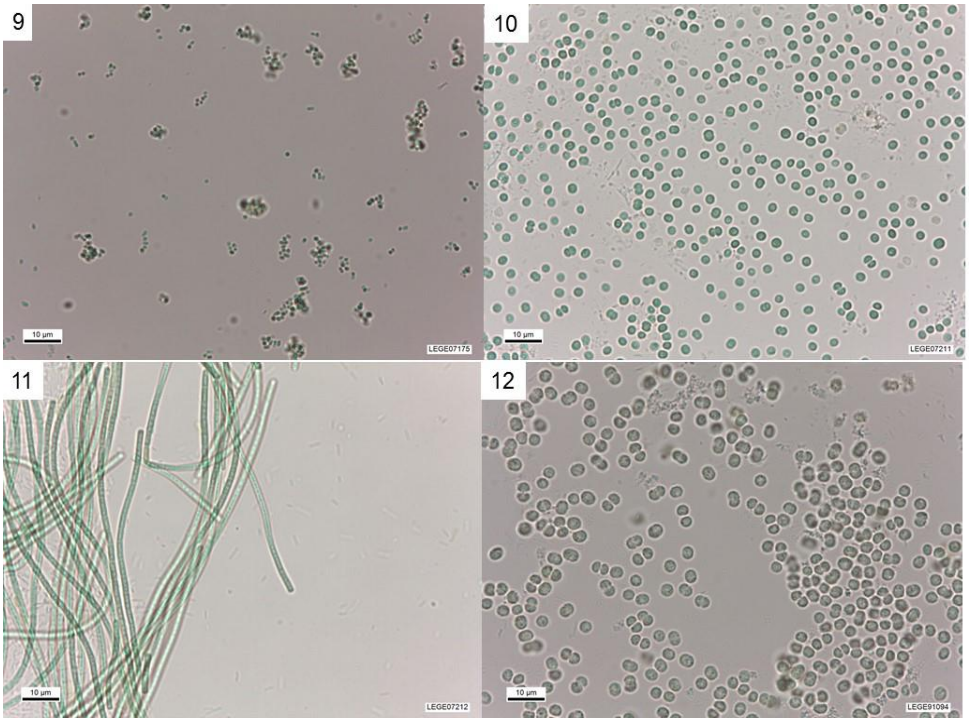


Figure 16: Microscopy's view of cyanobacteria strains. 9: LEGE 07175; 10: LEGE 07211; 11: LEGE 07212; 12: LEGE 91094 (From BBE collection)

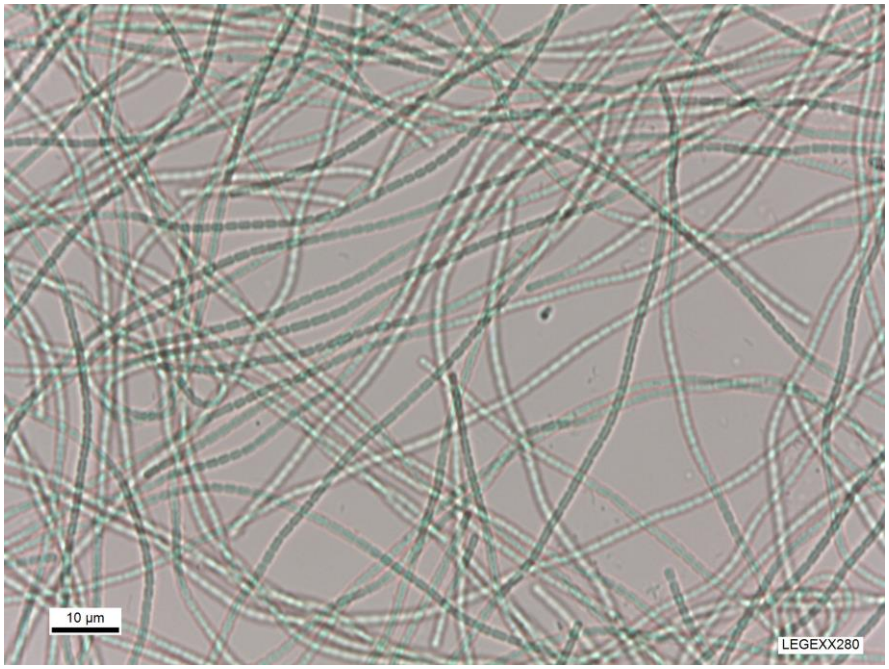


Figure 17: Microscopy's view of cyanobacteria strain LEGE XX280 (From BBE collection)

Cyanobacterial strain	Strain (LEGE)	Extract	Fractions (10 mg/ml)	Total
Synechocystis sp.	07211	14031	A-I	9
Limnothrix sp.	07212	14067	A-J	10
Oscillatoria limnetica	00237	14032	A-J	10
Planktothrix planctonica	XX280	14028	A-I	9
Aphanizomenon sp.	03283	14035	A,B,D,E,G,I,J	7
Phormidium sp.	06363	14026	A,C,D,E,F,G,H,I,J,K	10
Leptolyngbya sp.	07075	15093	A-I	9
Synechocystis sp.	06079	13003	A,C,D,E,F,G,H	7
Microcystis aeruginosa	91094	13004	A,B,D,E,F,G,H,I	8
Nodularia sp. *(*aff. *Sphaerocarpa/harveyana)*	06071	13005	A-I	9
Leptolyngbya sp.	07084	13008	A-I	9
Nostoc sp.	06077	13013	A-J	10
Cyanobium sp.	07175	16112	A-J	10

Table 5: Cyanobacteria strains and respective fractions that were tested in the biological assays

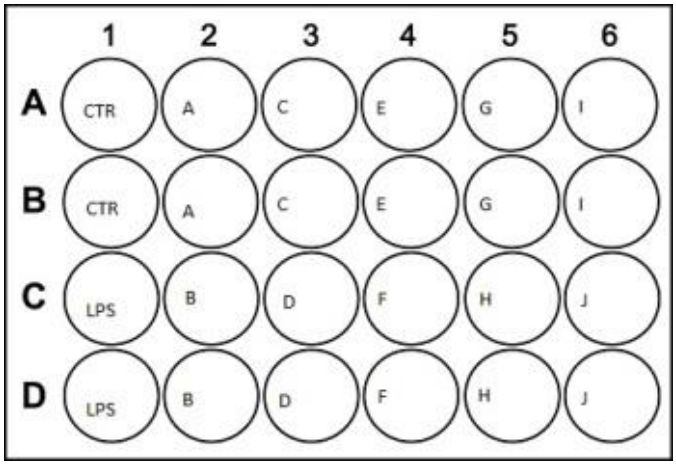


Figure 18: Example of the exposure of an extract and respective fractions

After 24 hours of incubation, the samples were subjected to the process described in 3.3.1 and NO levels in supernatant were quantified. The amounts of NO produced for each treatment were obtained by reading the absorbance in a spectrometer with the wavelength of 540 nm, and by extrapolating to values from a calibration curve using a gradient of concentration of sodium nitrite. The NO values were obtained through the equation of the calibration curve, in $Y = mx + b$.

3.5 Column chromatography (by gravity) of E14026 A

After the biological assays, the fraction E14026 A revealed potential bioactivity, and was subjected to a column chromatography (silica gel 60, 0.0015-0.040 mm), Merck, KgaA, Damstadt, Germany) (Figure 19). This fraction had a weight of 69.5 mg. As this fraction is the most polar of all fractions, the silica column was prepared with 100% of hexane. Fraction E14026 A was resuspended in EtOAc in ethyl acetate with the help of ultrasounds, and loaded on top of the silica column. The gradient of solvents was added stepwise, according to Table 6. Through this process, 72 samples were collected in 10 ml tubes. Thin layer chromatography (TLC) (silica 60 F254 (Merck)) was performed to visualize samples under UV light in order to assess the efficiency of the chromatography and to extrapolate the composition of each mixture, as elucidated in Figure 20. Due to similar composition, (from the TLC analysis), some samples in elution tubes were pooled as depicted in Table 7. In that way, 8 sub-fractions were obtained (1-8) and evaporated in RB flasks. The sub-fractions were dried in a rotary evaporator, re-suspended, transferred to pre-weighed vials, dried and weighed (see Figure 21). The obtained fractions were subsequently tested in the biological assays.

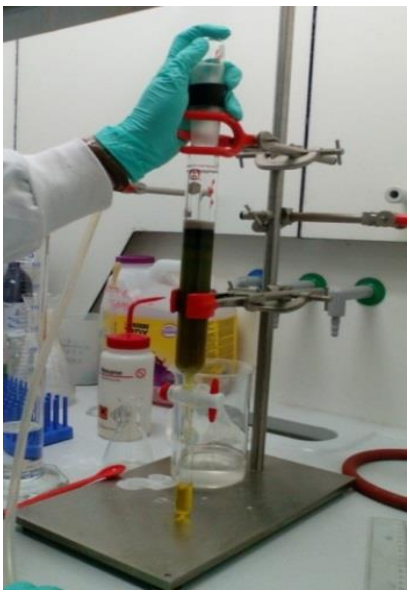


Figure 19: Column chromatography of the fraction LEGE 06363 - E14026 A

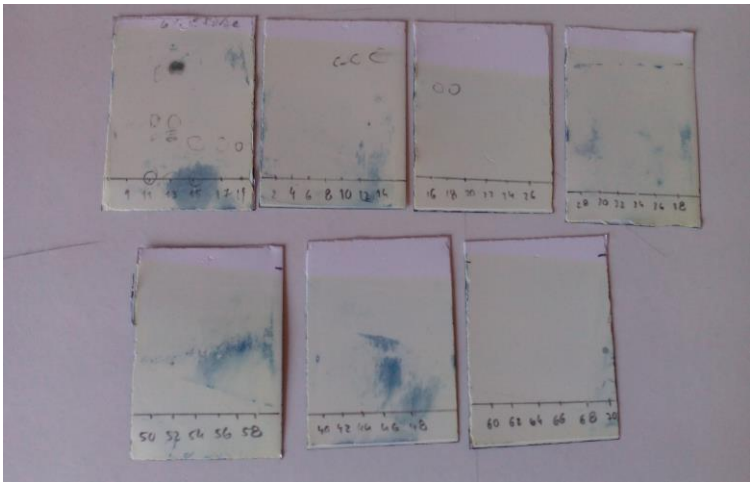


Figure 20: TLC of the samples collected during the chromatography process of E14026 A

Figure 21: Rotary evaporator for dry the samples and fractions obtained after chromatography

5% EtOAc	95% Hexane
6% EtOAc	94% Hexane
8% EtOAc	92% Hexane
10% EtOAc	90% Hexane
12% EtOAc	88% Hexane
15% EtOAc	85% Hexane
20% EtOAc	80% Hexane
100% EtOAc	-----
9% Methanol	91% EtOAc

Table 6: Gradient of solvents in chromatography

Sub-Fraction	Tubes (10 mL)	RB Flasks
E14026 A1	1 - 9	500 mL
E14026 A2	10 - 12	100 mL
E14026 A3	13 - 14	100 mL
E14026 A4	15 - 20	200 mL
E14026 A5	21 - 44	500 mL
E14026 A6	45 - 67	500 mL
E14026 A7	68 - 70	100 mL
E14026 A8	72 + erlenmeyer	200 mL

Table 7: 8 sub-fractions obtained after the TLC

3.6 Flash Chromatography – Solid Phase Extraction (SPE) of E14035 A

Fraction A from the extract E14035 revealed strong activity in the biological assays. So with a weigh of 173.8 mg, it was decided to perform a flash chromatography using SPE.

The column was a normal phase column SPE (Si-20g, Strata, Phenomenex) and silica column was compacted with 100% Hexane. The fraction A was resuspended in 10% Ethyl Acetate (EtOAc) and 90% Hexane and loaded in the silica column. The mixtures of solvents were carefully added stepwise, according to Table 8 and 53 samples were collected in 10 mL tubes. Thin layer chromatography (TLC) (silica 60 F254 (Merck)) was performed for every eluate and visualized under UV light in order to assess the efficiency of the chromatography and to extrapolate the composition of each mixture, as elucidated in Figure 23. Some samples were pooled due to assumed similar composition as elucidated in Table 9. Sub-fractions were established and evaporated in RB flasks. These mixtures were dried in a rotary evaporator, resuspended, transferred to pre-weighed vials, dried and weighed before tested in biological assays.

0% EtOAc	100% Hexane
1% EtOAc	99% Hexane
2% EtOAc	98%Hexane
4% EtOAc	96% Hexane
6% EtOAc	94% Hexane
8% EtOAc	92% Hexane
10% EtOAc	90% Hexane
20% EtOAc	80% Hexane
40% EtOAc	60% Hexane
100% EtOAc	-----
0.1% TFA	99,9% EtOAc

Table 8: Gradient of solvents in chromatography

Sub-Fraction	Tubes (10 mL)	RB Flasks
E14035 A1	1 - 11	500 mL
E14035 A2	12 - 21	500 mL
E14035 A3	22 - 25	100 mL
E14035 A4	26 - 27	100 mL
E14035 A5	28 - 29	100 mL
E14035 A6	30 - 34	200 mL
E14035 A7	35 - 39	200 mL
E14035 A8	40 - 53	500 mL

Table 9: 8 sub-fractions obtained after the TLC

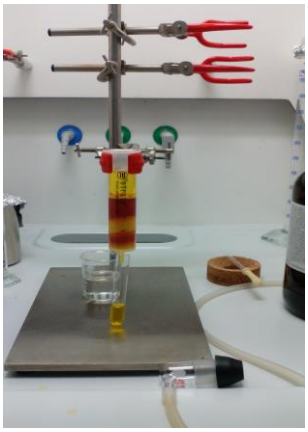


Figure 22: SPE chromatography of E14035 A

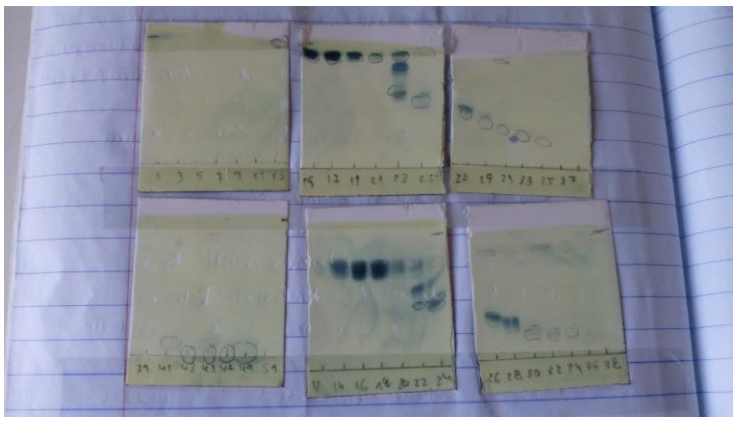


Figure 23: TLC of the sub-fractions of E14035 A collected during the chromatography process

3.7 Semi-preparative normal-phase High Performance Liquid Chromatography of E14035 A2

After the biological assays, sub-fraction A2 was selected for further purification using the semi-preparative HPLC, a 1525 Binary HPLC pump and a UV-Vis detector (Waters, Milford, MA, USA). This process requires an optimization before the chromatography and therefore it is used a Luna 5u Silica (2) column (250 x 4.6 mm, 100 A, Phemomenex) in order to assure maximum possible separation of compounds. The Table 10 shows the selected conditions, following a gradient of solvents, after the optimization process. Previously, the fraction E14035 A2 was resuspended in a mixture of chloroform and hexane. The obtained spectrum - the chromatogram - allowed us to collect the compounds, according to the observed peaks, in RB flasks from multiple injections of 500 µl each before evaporation in the rotary evaporator. Sub-fractions were re-suspended, transferred to pre-weighed vials, dried and weigh. Finally, we obtained 11 new sub-fractions. These 11 fractions were tested in the bioassay to evaluate anti-inflammatory properties, before 1H NMR (400 MHz, BrukerAvance III) analysis.

Time (min)	Flow (mL/min)	EtOAc	Hexane
0	3	0%	100%
28	3	20%	80%
33	3	50%	50%
40	3	50%	50%
43	3	0%	100%
48	3	0%	100%

Table 10: Mobile phase gradient used for the semi-preparative HPLC on sub-fraction E14035 A2

Extract	Sub-Fraction	Weight (mg)
E14035	A2 A	0.83
	A2 B	2.17
	A2 C	1.13
	A2 D	1.3
	A2 E	0.66
	A2 F	0.4
	A2 G	0.77
	A2 H	1.23
	A2 I	2.56
	A2 J	8
	A2 K	1.95

Table 11: Weight of sub-fractions of E14035 A2 after HPLC

3.8 Semi-preparative normal-phase High Performance Liquid Chromatography of E14035 A2 J

After the biological assays and regarding to the weight of each sub-fraction and the results of 1H NMR, the fraction E14035 A2 J was chosen for further purification. In this case, it was utilized an analytical column - Luna 5u Silica (2) 250 x 4.6 mm, 100 A to optimize the procedure and to separate the compounds. The Table 12 shows the selected conditions, following a gradient of solvents, after the optimization process. For this sample, the optimization took some time, since it was necessary to try different percentages of the solvents and different gradients to perform the best separation. Previously, the fraction E14035 A2 J was re-suspended in a mixture of ethyl acetate and hexane. The obtained spectrum - the chromatogram - allowed us to collect the compounds, according to the observed peaks, in RB flasks from multiple injections with 100 µl each one and then evaporated in the rotary evaporator. Sub-fractions were re-suspended, transferred to pre-weighed vial, dried and weighted. Finally, we obtained 13 new sub-fractions. These 13 fractions were tested in the bioassay to evaluate anti-inflammatory properties, before 1H NMR (400 MHz, BrukerAvance III) and LC-MS analysis.

Time (min)	Flow (mL/min)	EtOAc	Hexane
0	1	5%	95%
8	1	5%	95%
50	1	40%	60%
55	1	60%	40%
60	1	60%	40%
65	1	5%	95%
70	1	5%	95%

Table 12: Mobile phase gradient used for the semi-preparative HPLC on sub-fraction E14035 A2J

Extract	Fraction	Weight (mg)
E14035	A2J1	1,18
	A2J2	0,57
	A2J3	-
	A2J4	0,22
	A2J5	0,09
	A2J6	0,34
	A2J7	0,03
	A2J8	0,62
	A2J9	0,09
	A2J10	0,44
	A2J11	0,29
	A2J12	0,59
	A2J13	3,57

Table 13: Weight of sub-fractions of E14035 A2J after HPLC. No weight was obtained for subfraction A2J3.

4. Results

4.1 Assay optimization

For the first months, the main goal was to setup the assay conditions and to optimize the process of inflammation, induced by LPS. Different concentrations of LPS were tested (100 ng/mL, 50 ng/mL and 25 ng/mL) and different designs were tested: 96 well-microplate vs 24-well microplate. The Figure 24 shows one example of the assay in a 96-well microplate that did not work perfectly, since LPS-stimulation did not work well. In several trials, we could not induce NO production in the 96-well format, but only in the 24-well format. Consequently, we used the 24-well format for all following assays. The induction of inflammation had to be improved through several assays, since in the beginning no consistent results were obtained. Finally, we increased the concentration to 1000 ng/mL LPS, which demonstrated consistent results and a stable induction of inflammation. Consequently, this concentration was selected for the bioassays with the extracts/fractions from cyanobacteria.

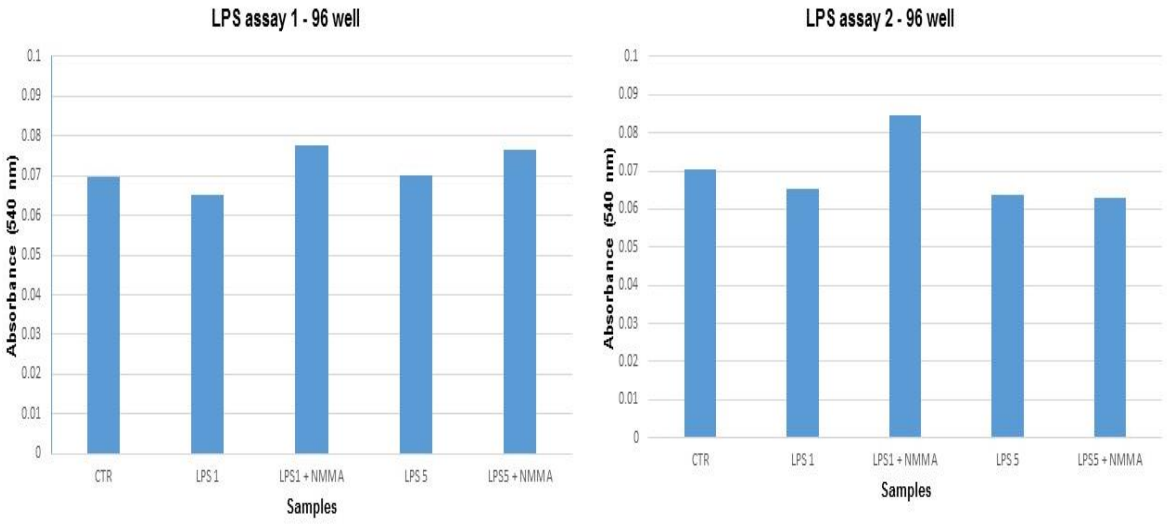


Figure 24: LPS assay performed with 1 μg/ml concentration of LPS in 96 well-microplate. In LPS assay 1 the LPS was pipetted directly into the medium, while in the assay 2 the LPS was first mixed with the medium and vortexed. (Final concentration: LPS1: 0.0099 μg/mL; LPS5: 0.048 μg/mL)

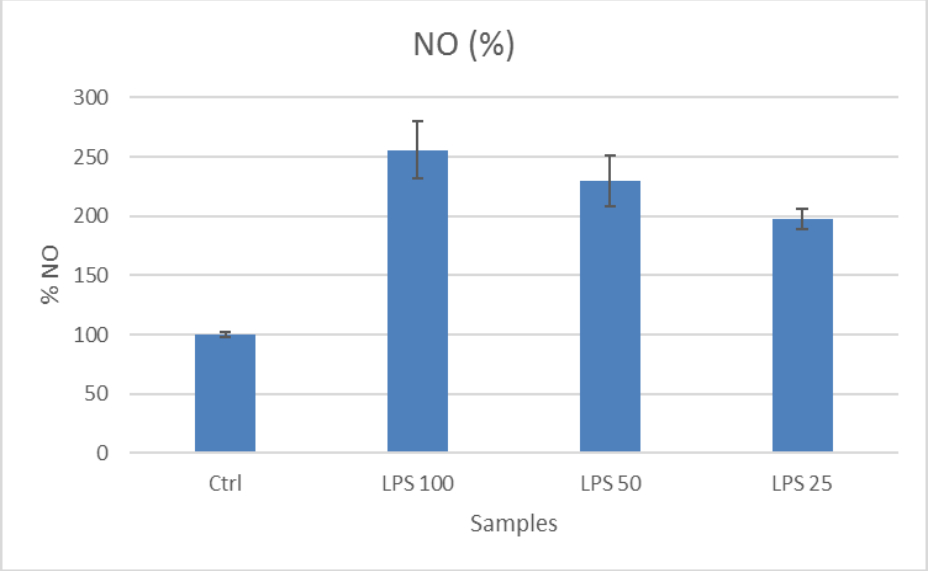


Figure 25: Example of a successful LPS assay in a 24-well microplate with different concentrations of LPS inducing the inflammation in the cells (LPS 100: 100 ng/ml; LPS 50: 50 ng/ml; LPS 25: 25 ng/ml). The graphic shows the % of NO production in the culture supernatant measured by the Griess Reaction at 540 nm.

4.2 Screening assays for cyanobacterial activity

The extracts used during the present work were obtained in a previous project (MarBiotech), following the standard procedure of the BBE group in CIIMAR. Cyanobacterial biomass was submitted to a normal phase vacuum liquid chromatography which resulted in 9 - 11 fractions.

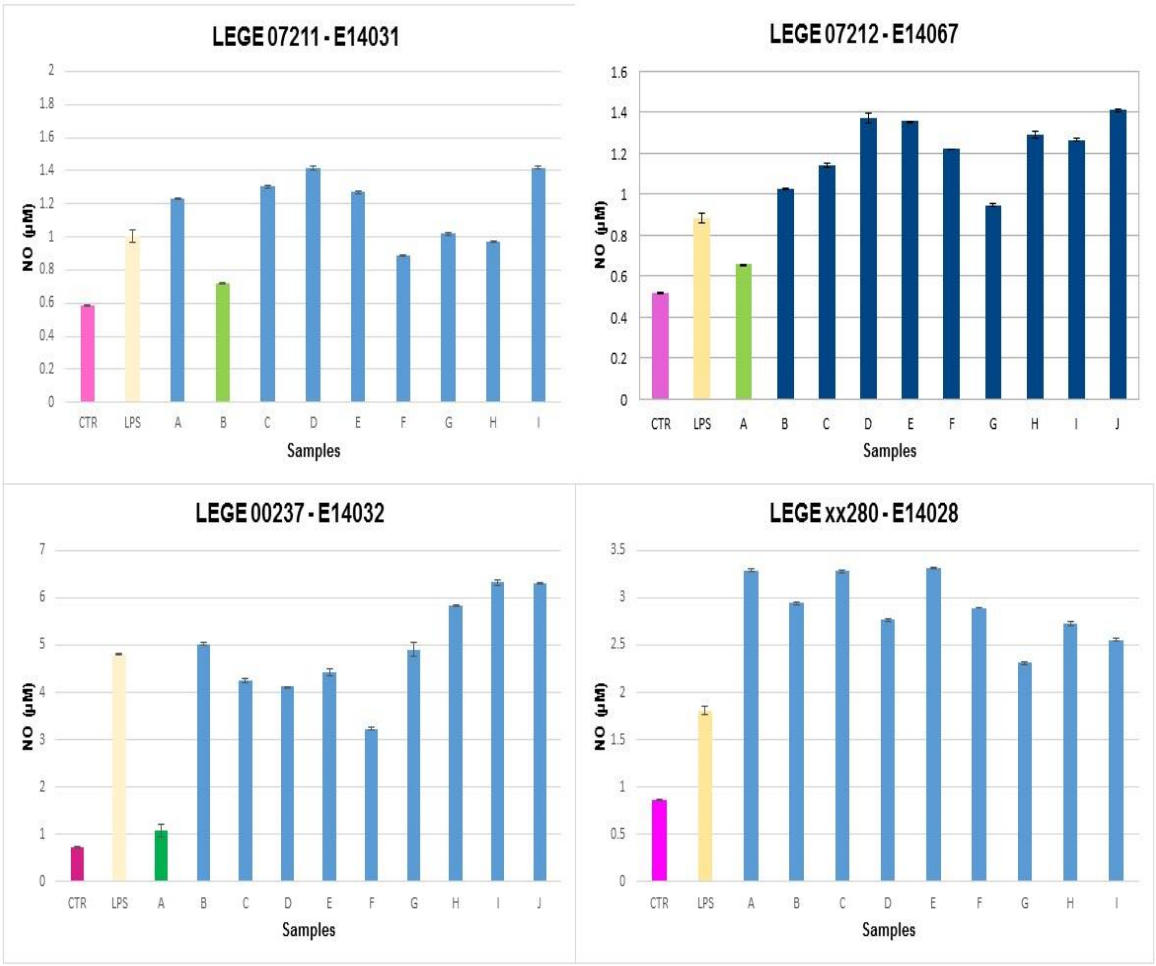


Figure 26: Representation of the results relative to the strain LEGE 07211, LEGE 07212, LEGE 00237 and LEGE XX280 and respective fractions A-I in NO units, after 24 h of exposure. CTR: 0.3 % of DMSO, LPS: 1 µg/ml. Fractions were co-exposed to the same LPS concentration. Two replicate wells were assayed for each group.

In our assay, we used at least 2 replicate wells for each treatment, which allowed us to have a maximum of 12 different groups in a 24-well plate. The assay design included 2 replicate wells for the solvent control (0.3% of DMSO), for LPS (1 µg/ml) with 0.3% of DMSO, and for each fraction.

The activity of each fraction of the strain LEGE 07211 is shown in NO units. Regarding to this, fraction E14031B revealed more anti-inflammatory activity. This extract was able to reduce the induction of inflammation by LPS, from 1 µM to 0.7 µM (1.4 fold), a similar level as the solvent control (0.6 µM). The strain LEGE 07212 had one fraction that showed strong potential in anti-inflammatory activity – E14067A, which inhibited the LPS induced NO production (0.9 µM) to the similar level as the solvent controls cells in this assay (0.6 µM). The other fractions did not reduce the LPS-induced NO production, but increase the inflammatory process (Figure 26). LEGE 00237 showed strong anti-inflammatory

potential, since one fraction (E14032A) decreased effectively the LPS-induced NO production. The reduction of NO production was about 5fold less compared to the LPS treatment (LPS 5 µM, E14032A 1 µM). The fractions of strain LEGE XX280 did not show any anti-inflammatory activities.

Most of the fractions of the strains LEGE 06079, LEGE 91094, LEGE 06071 and LEGE 07084 did not show significantly results regarding to anti-inflammatory activity (Figure 27).

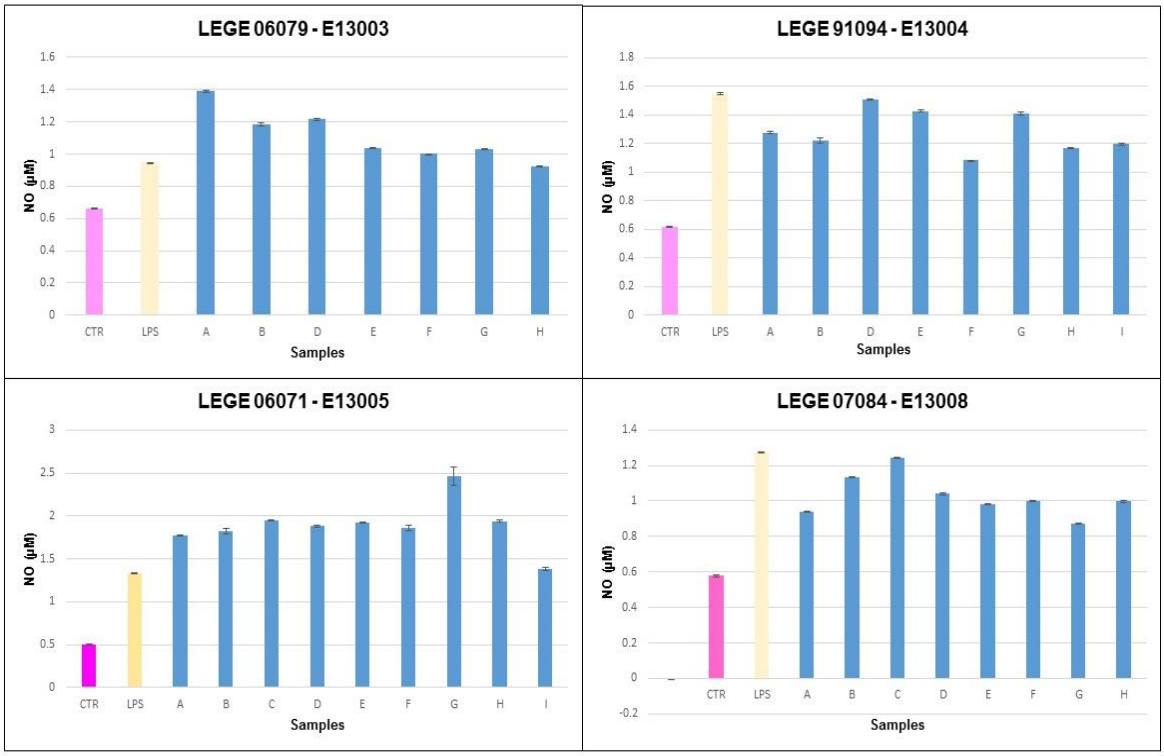


Figure 27: Representation of the results relative to the strain LEGE 06079, LEGE 91094, LEGE 06071 and LEGE 07084 and respective fractions A-I in NO units, after 24 h of exposure. CTR: 0.3 % of DMSO, LPS: 1 µg/ml. Fractions were co-exposed to the same LPS concentration. Two replicate wells were assayed for each group.

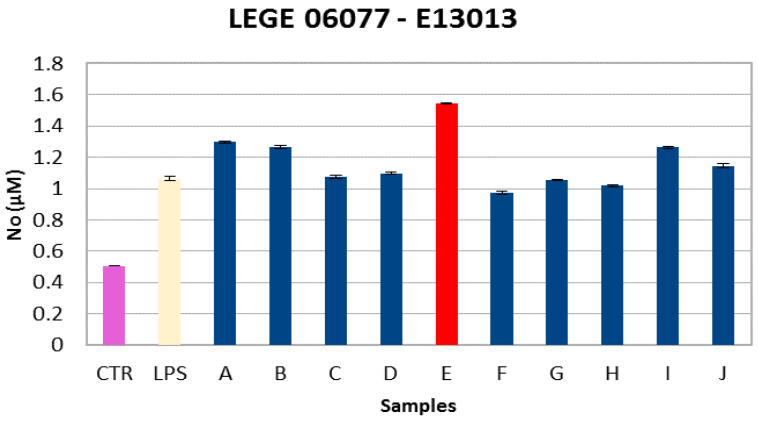


Figure 28: Representation of the results relative to the strain LEGE 06077 and respective fractions A-J in NO units, after 24 h of exposure. CTR: 0.3 % of DMSO, LPS: 1 µg/ml. Fractions were co-exposed to the same LPS concentration. Two replicate wells were assayed for each group.

The analysis of the fractions from the strain LEGE 06077 - E13013 showed that fraction E had a pro-inflammatory activity, with a NO production of 1.5 µM, compared to a NO production of 1 µM, on the LPS treatment (Figure 28).

The analysis of the fractions from LEGE 06363 from the cyanobacteria collection revealed strong anti-inflammatory activity for fractions A, C and G, while pro-inflammatory activity was observed in fraction D.

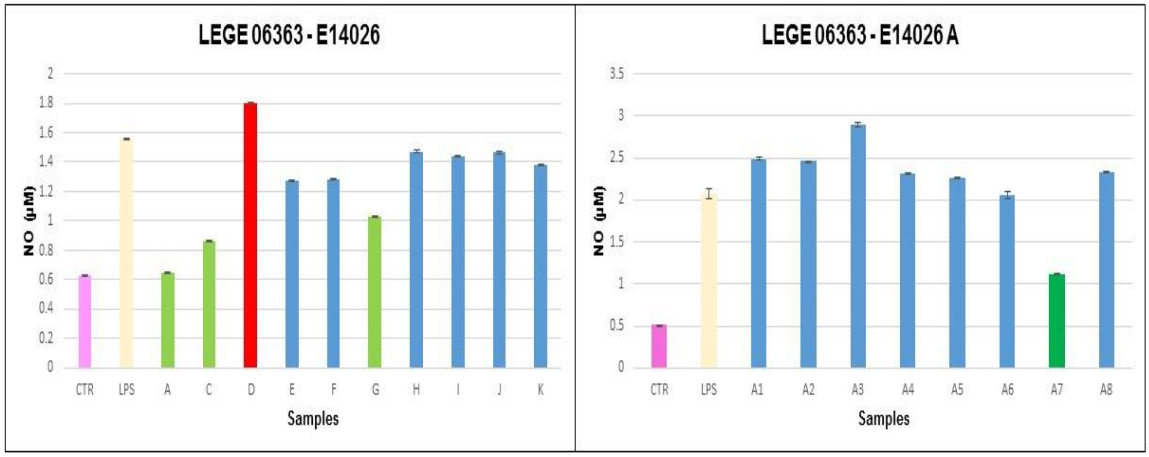


Figure 29: Representation of the results relative to the strain LEGE 06363 and respective fractions in NO units, after 24 h of exposure. CTR: 0.3 % of DMSO, LPS: 1 µg/ml. Fractions were co-exposed to the same LPS concentration. Two replicate wells were assayed for each group. The right panel shows the results for the sub-fractions of fraction E14026A.

After the column chromatography of the fraction LEGE 06363 A, the obtained sub-fractions were re-tested. The sub-fraction A7 showed strongest anti-inflammatory activity (Figure 29). The subsequent NMR analysis demonstrated that the sub-fraction A7 still contained some complexity and would need further purification steps to isolate the responsible compound (Figure 30). However, due to the relatively low biomass of 1 mg, it was decided to continue the bioassay-guided isolation of novel compounds with another fraction that showed promising anti-inflammatory activity, but had a higher biomass available. More biomass will be needed in the future, in order to isolate the responsible compound.

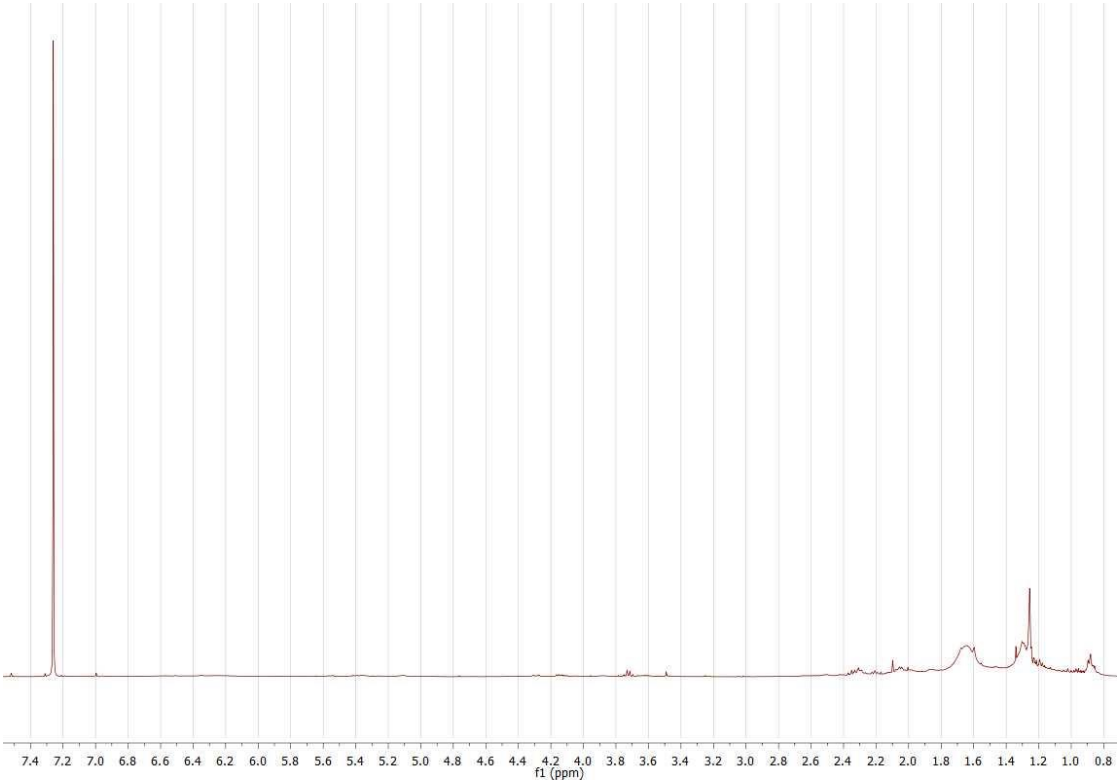


Figure 30: ^{1}H NMR of the fraction E 14026 A7

The fraction A from the cyanobacterial strain LEGE 03283 showed strong anti-inflammatory activity (Figure 31). After flash chromatography of this fraction with an initial weight of 173.8 mg, the obtained sub-fractions were re-tested. Clearly, the sub-fraction A2 (E14035 A2) displayed the strongest anti-inflammatory activity. Following, the sub-fraction E14035 A2 with 20.66 mg was submitted to further purification by HPLC, as explained in the material and methods part.

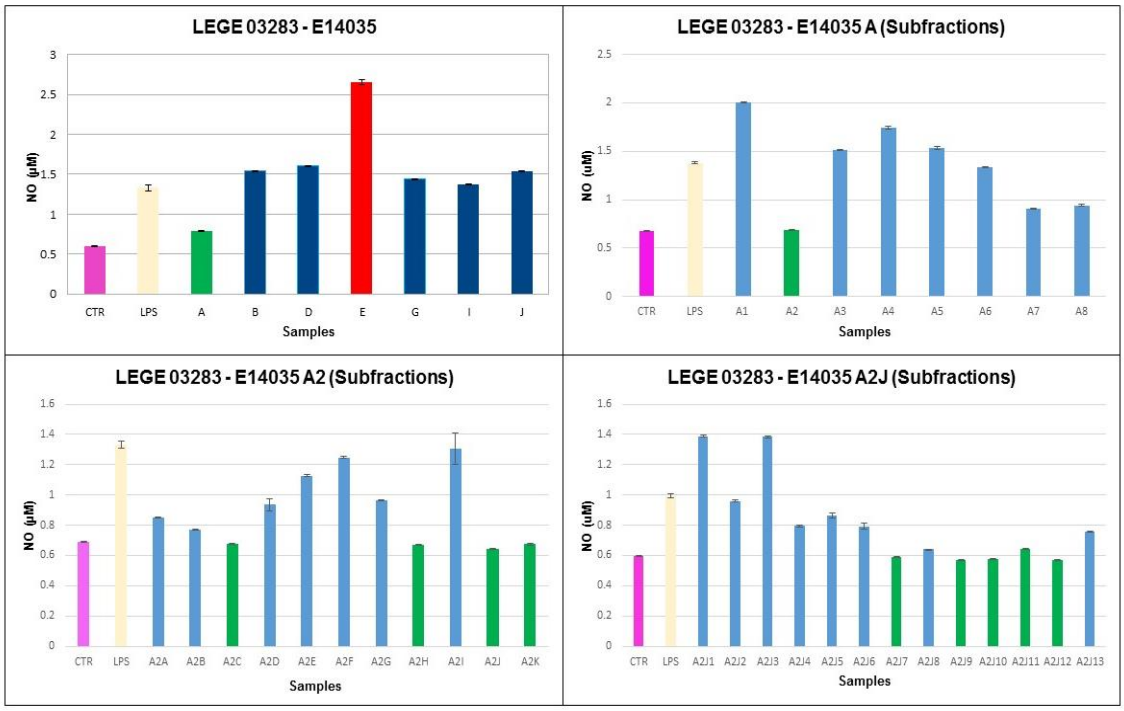


Figure 31: Representation of the results relative to the strain LEGE 03283 and respective fractions in NO units, after 24 h of exposure. CTR: 0.3 % of DMSO, LPS: 1 µg/ml. Fractions were co-exposed to the same LPS concentration. Two replicate wells were assayed for each group. The panels show the results for activity screening of the sub-fractions.

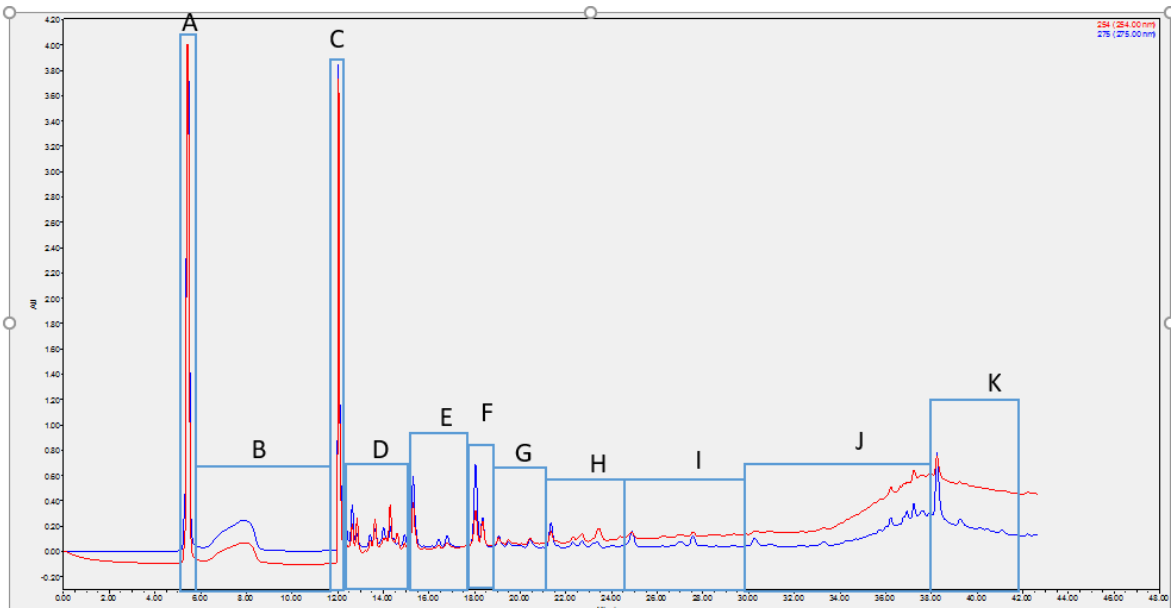


Figure 32: Chromatogram of E14035 A2 showing highlighted sub-fractions that were collected. Conditions of the injection were 500 µL at a concentration of approximately 4.5 mg mL-1.

The fractions A2C, A2H, A2J, and A2K from the cyanobacterial strain LEGE 03283 were the most active ones in the biological assays using the Griess method. In the ^{1}H NMR

spectrum, the sub-fraction A2J contained just a few compounds, and further purification were necessary.

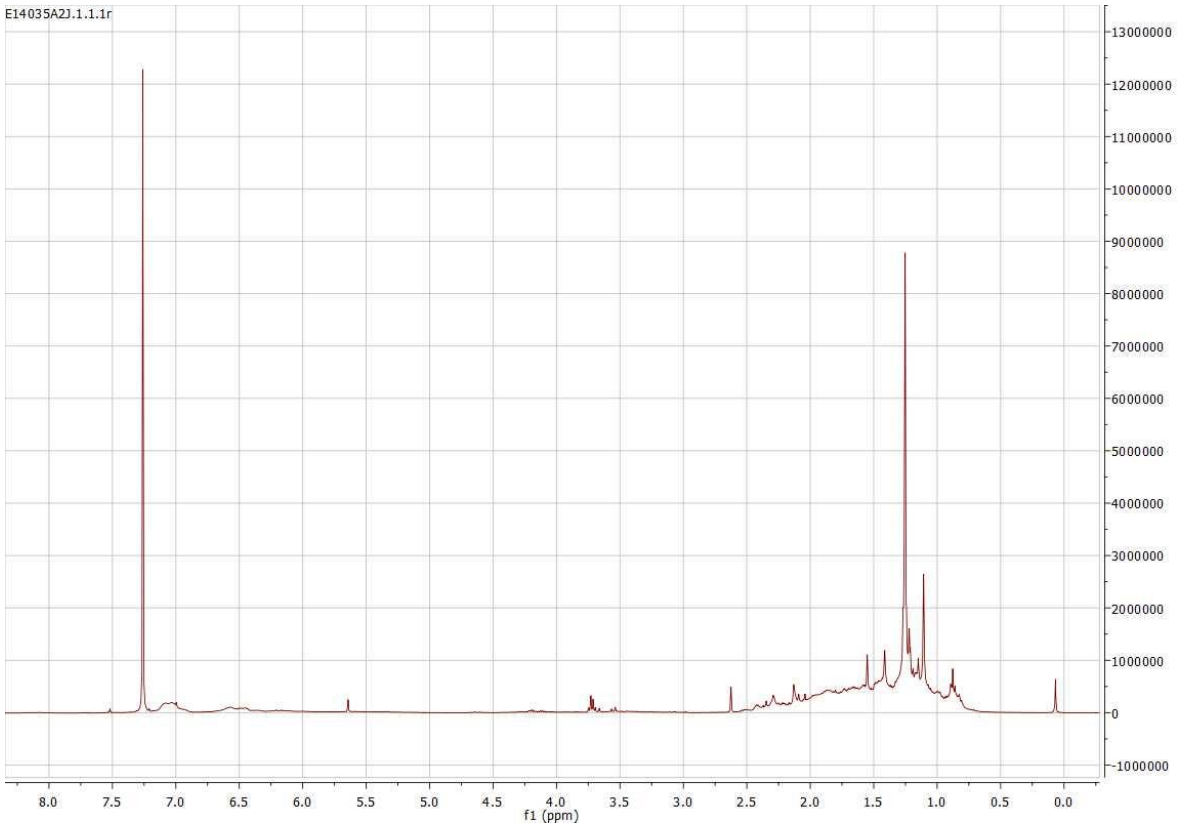


Figure 33: ^{1}H NMR of the fraction E 14035 A2 J

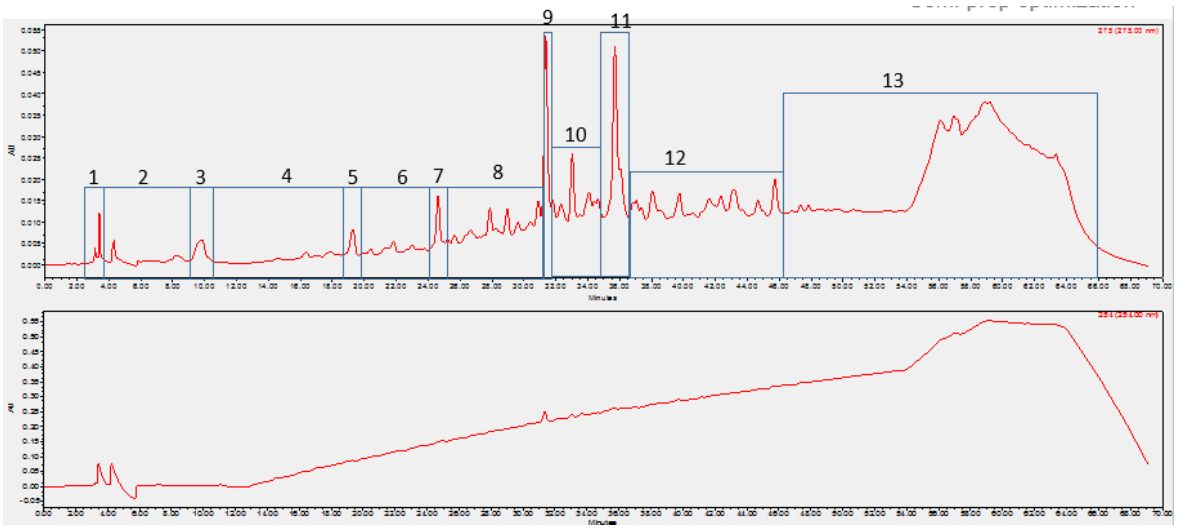


Figure 34: Chromatogram of E14035 A2J showing highlighted sub-fractions that were collected. Conditions of the injection were 100 µL at a concentration of approximately 4.5 mg mL-1. The detection was made at 275 nm and 254 nm.

The HPLC separation of the sample E14035 A2J was performed subsequently with 100 µL loaded each time. Each run had a period of time of 70 minutes and it was necessary to perform about 20 runs to separate the whole sample. The signal detection of different compounds was better at 275 nm wavelength compared to 254 nm wavelength.

After HPLC purification, 13 fractions were obtained and tested for anti-inflammatory activity. Fractions A2J7, A2J8, A2J9, A2J10, A2J11 and A2J12 revealed to be the most active ones (Figure 31). A2J7, A2J9, A2J10, A2J11 and A2J12 were subjected to a ^{1}H NMR analysis to check their complexity (Figure 35).t

These subfractions contain still two or more compounds, therefore further purification by HPLC will be necessary.

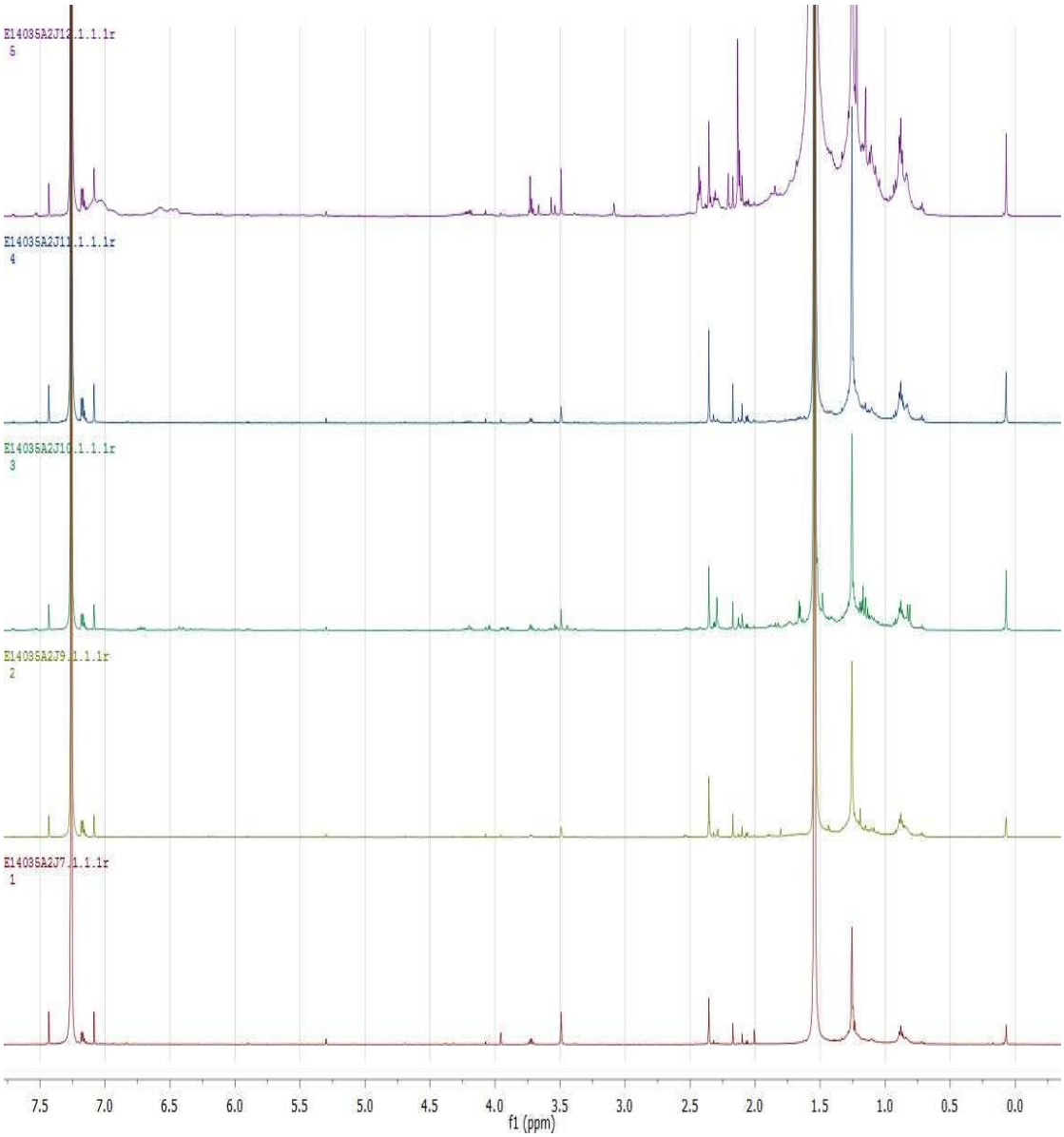


Figure 35: ^{1}H NMR of the fraction E 14035 A2J7 (on the bottom), A2J9, A2J10, A2J11 and A2J12 (on the top) (recorded at 600 mHz).

Strain	Fraction	Anti-inflammatory activity
LEGE 07211	E14031 B	++
LEGE 00237	E14032 A	++
LEGE 06363	E14026 A7	++
LEGE 03283	E14035 A2J	++

Table 14: Summary of anti-inflammatory activity from all tested cyanobacterial strains

5. Discussion

As explained previously in this work, inflammation is a process that happens as a response of the immune system of mammals to protect them against infections derived from bacterial or fungi (Kumar *et.al.*, 2015; Cruvinel *et.al.*, 2010). Be a chronic disease or an acute disorder, or even a light migraine, humans easily resort to synthetic drugs to treat the symptoms. The most used are NSAID's and act as a blockade of the mediators that are produced during the process of inflammation. (www.CRBestBuyDrugs.org, Rao & Knaus, 2008). Although the treatment of inflammation by NSAID's is effective, the constant use of these drugs can still bring many side effects such as the injury of the gastrointestinal and renal systems, fluid and salts retention, heart attacks, strokes, and others (Qandil *et.al.*, 2012; Gouda *et.al.*, 2013; Rao & Kaus, 2008). Because NSAID's can manifest adverse side effects (Fitzgerald, 2004), one effort has been to identify natural products, derivate from plants, microalgae and cyanobacteria for the prevention and treatment of inflammatory disorders.

LPS optimization

Before all the bioassays with the cyanobacterial extracts, we had to optimize the method of induction of inflammation in the cells. We used LPS, the major component of the gram negative bacteria, which induces inflammation and therefore stimulate the NO production by macrophages. We tested different concentrations and different ways of performing the assay. Most of the works previously published in this matter, indicated that the best concentration is 1 μg/ml to induce inflammation and NO production in RAW 264.7 cells (Aldrige *et.al.*, 2008; Damte *et.al.*, 2011; Dey *et.al.*, 2005, Kellog *et.al.*, 2015; Yang *et.al.*, 2014; Yang *et.al.*, 2012). In BV2 microglia, the inflammatory response required a lower LPS' concentration and 100 ng/ml was frequently used (Henn *et.al.*, 2009). Olszanecki *et.al.* (2006) showed that LPS at 1 μg/ml caused a significant increase of NO accumulation, after 24 hours of incubation, in the macrophage cell line J744.2. The human cell line – Thp1 – has to be treated with 10 μg/ml of LPS to induce inflammation (Speranza, 2010).

In our assays, the best concentration was 1 μg/ml that consistently increased the NO production. The best conditions were to mix the LPS rigorous with the medium and to add directly to the microplate. If we added the LPS to the medium in the plates, sometimes the assay did not work well. We also tried to induce NO production by LPS in 96 well-microplates, however without success. In contrast, other works with RAW 264.7 cells

demonstrated that assays were performed in 96 well-microplate with good and expected results (Damte *et.al.*, 2011; Villa *et.al.*, 2010 (b); Yang *et.al.*, 2014). The reasons for this discrepancy are not clear yet. One part of the explanation could be that LPS is an apolar molecule; the dissolution of LPS is not easy, and additionally 96 well-microplate has more plastic area in direct contact with LPS. We suppose that the LPS may interact with the plastic of the microplate, which will reduce its concentration in the medium. However, inflammation assays in 24 well-microplate were more common in this cell line that in 96 well-microplate (Dey *et.al.*, 2005; Jo *et.al.*, 2010; Kellog *et.al.*, 2015; Oliveira *et.al.*, 2014; Yang *et.al.*, 2012).

Cyanobacterial strains with anti-inflammatory activity

In this work, we tested 117 fractions from 13 marine cyanobacteria strains of different classes for their anti-inflammatory activity. The chosen strains have already shown previous activity in other studies like in an adipocytes cell line. Fractions of *Planktothrix planctonica* LEGE XX280 (E14028 I) and *Aphanizomenon* sp. LEGE 03283 (E14035 B) exerted strong positive effects in proliferative and metabolic activity of 3T3-L1 pre-adipocytes. Fractions from *Synechocystis* sp. LEGE 07211 (E14031 D) and from *Oscillatoria limnetica* LEGE 00237 (E14032 H and J) showed remarkable activity in the modulation of adipogenesis of murine pre-adipocytes (Castro, 2016). It should be generally noted that different fractions of the same strains had anti-inflammatory activities or anti-obesity activities.

However, in some cases a concordance was observed, meaning that fractions had both, anti-inflammatory and anti-obesity activities. From *Synechocystis* sp. LEGE 07211, we obtained 9 fractions, and E14031 B reduced the LPS-induced NO production. The same fraction strongly inhibited adipogenesis in 3T3-L1 cells in a previous study (Castro, 2016) and now has a strong potential in anti-inflammatory activity.

The E14067 A from *Limnothrix* sp. LEGE 07212 revealed anti-inflammatory activity, reducing NO production about 25%. C-phycocyanin is a blue photosynthetic accessory pigment that was firstly isolated from *Spirulina*, accounting for 14% of the cyanobacteria's dry weight. C-phycocyanin exerted strong anti-inflammatory effects in Kupffer cells from mice liver through the inhibition of TNF-α response after induction of thyroid calorigenesis, and was able to reduce colonic injury and inflammatory cell infiltration in rats with acetic acid induced colitis (Castro *et.al.*, 2016). Other studies revealed that phycocyanin is a powerful antioxidant agent (Gantar *et.al.*, 2012) and that some other cyanobacterial

genera can be a source of this compound. In this study, the authors isolated the bioactive compound from the strain *Limnotrhrix* sp. 37-2-1 and then evaluate its antioxidative activity by electron spin resonance (ESR) spectroscopy, which showed 100% activity at 0.15 mg/ml (Gantar *et.al.*, 2012). We can hypothesize that C-phycocyanin is the compound present in the tested strain *Limnothrix* sp. LEGE 07212 and it is interacting with some pathway involved in the inflammatory response, explaining, thus, the fact that its fraction has anti-inflammatory activity.

Researchers have pointed to more complex bacteria (from orders Oscillatoriales, Nostocales or Stigonematales) as more prolific in secondary metabolites. However, our bioassays show that simpler cyanobacteria, like *Synechocystis* sp. LEGE07211 from the sub-class Chroccocales, may also possess novel secondary metabolites with pharmaceutical and biotechnological potential.

The strain *Oscillatoria limnetica* LEGE 00237 showed strong evidence in having anti-inflammatory activity for the fraction E14032 A with a reduction of the NO production from 4.82 ± 0.015 μM in the LPS' treatment to 1.08 ± 0.14 μM (5 fold less than in the LPS treatment). Although this strain and fraction in particularly exerted strong effects on the RAW 264.7 cell line, we could not evaluate its bioactive potential, because of the low available biomass – 36.5 mg – that was not enough to proceed with the purification and isolation of the responsible compound.

The strain *Phormidium* sp. LEGE 06363 with the extract E14026 from the cyanobacteria collection revealed some bioactive fractions: A, C and G with anti-inflammatory activity and one (E14026 D) with pro-inflammatory activity (Figure 36). Ulivi *et.al.* (2011) already revealed that an isolated compound – monogalactosyldiacylglycerol (MGDG) - of the cyanobacterial strain *Phormidium* sp. repressed the inflammatory response induced in human articular cells via IL-1α + TNF-α without impairment of cell viability. In this case, the main target of MGDG were the IL-6 and its soluble receptor, since they are related in inflammatory and degenerative diseases. Increased levels of IL-6 and sIL-6R were found in synovial fluids and sera from osteoarthritis and rheumatoid arthritis patients and their level correlates with the increased leukocyte infiltration in synovial tissue. In *in vitro* assays, this compound exerted strong anti-inflammatory activity in cultured articular chondrocytes through the p38 and NF-KB pathways inhibition. In addition, MGDG could be able to activate an anti-inflammatory pathway involving $15\Delta PGJ_2$. Another strain of this class and order Nostocales – *Phormidium tenue* – produced several diacylglycerols that

inhibited chemically induced tumours on mice and was reported as HIV inhibitor (Singh *et.al.*, 2005).

Regarding to our results, it was decided primarily to perform a column chromatography by gravity to separate the compounds present in the mixture of the E14026 A with a weight of about 69.5 mg. This fraction was the most non-polar, and through the gradient of solvents used for this chromatography 8 sub-fractions were obtained. Of this 8 sub-fractions, one reveal to be the most active one in the biological assays – E14026 A7. For this fraction, we performed the ^{1}H NMR analysis and as demonstrated in the results, the fraction is not isolated and it is still constituted by several different compounds. NMR is used as a screening technique in several research projects, for both comparison of spectral data found in literature or between fractions. Nuclear magnetic resonance has been a widely used method, extremely important, aiding the bioactivity guided fractionation process and structure elucidation of new metabolites (Pellecchia *et.al.*, 2002; Oldoni *et.al.*, 2016). Throughout this work, we used ^{1}H NMR to evaluate the separation procedure, since different NMR spectra are obtained for each fraction. Once the compounds are pure, 1D and 2D NMR can be used to elucidate the structure of the compounds.

Due to the low amount of biomass in the sub-fraction E14026 A7 (0.96 mg), it was not possible to carry on the isolation and purification of the compound with anti-inflammatory activity. Considering this result, it would be interesting to initiate a new cyanobacteria culture to grow the strain LEGE 06363 in a large scale in order to continue the sub-fractionation of the E14026 A7 and to isolate the compound in the future.

The fraction E14035 A from *Aphanizomenon* sp. LEGE 03283 from the order *Nostocales* revealed strong anti-inflammatory activity. E14035 A inhibited the 44% of the NO production, from 1.33 ± 0.038 μM (in the LPS treatment alone) to 0.79 ± 0.003 μM. This strain already showed bioactivity (E14035 B) in other assays, and demonstrated strong effects in proliferative and metabolic activity of 3T3-L1 pre-adipocytes (Castro, 2016). The strain *Aphanizomenon flos-aquae* is a type of cyanobacteria that are known to produce natural products rich in essential amino acids, c-linolenic acid (GLA), fibers, B vitamins, calcium, phosphorous, iron, pigments such as b-carotene, chlorophyll, xanthophylls, and others (Ku *et.al.*, 2013). *Aphanizomenon flos-aquae* is also known to produce several cyanotoxins that can be harmful to human and animals (Preuβel *et.al.*, 2006, Papendorf *et.al*., 1997), and it was reported that this strain is capable of producing antibiotics (Falch *et.al.*, 1995, Østensvik *et.al.*, 1998).

The extract E14035 A from LEGE 03283 was subjected to a SPE. This method was not perfect, and did not have a 100% of recovering of the biomass, because there was some disturbations in the solid phase. Probably, the silica was not well stabilized, thus in a certain moment of the chromatography, some compounds were not perfectly separated. The biological assays were performed again, and the fraction E14035 A2 showed to be the most active one with an inhibition of about 54% in NO production. According to the NMR analysis, sub-fraction E14035 A2 had some complexity with several compounds in its mixture. Following, HPLC allowed the separation of the fraction A2 (20.66 mg) into 11 new sub-fractions. From these 11 sub-fractions (A2A-A2K), three revealed anti-inflammatory activity. Through NMR analysis, it was possible to check the quantity of compounds still present in each sub-fraction and the complexity of each one. Once more, the NMR' spectrum showed that this compound was not pure yet and further purification was needed to isolate the bioactive compound. One bioactive sub-fraction – A2 J – had 8 mg after HPLC, and its NMR spectrum revealed that we still have a mixture of three or more compounds. This spectrum showed some instabilities, probably because the used vials were not completely dried and some solvents could have interacted with the ^{1}H NMR detection and behaved like a "mask". A2 J, in this case, was chosen to be submitted to a new separation by HPLC, in which we obtained 13 sub-fractions (A2J1-A2J13). From these 13 sub-fractions, five of them revealed anti-inflammatory activity – A2J7, A2J9, A2J10, A2J11 and A2J12.

In another study, a dichloromethane extract of *A. auricula-judae* inhibited LPS-induced NO production (1 μg/ml) in a dose-dependent manner in the concentration of ≥ 10 μg/ml ($p < 0.05$). The higher dose of *A. auricula-judae* extract (100 μg/ml) allowed a 4fold decrease of the NO production from about 16 μM (in the LPS treatment alone) to about 4 μM (Damte *et.al.*, 2011). In comparison, the fractions E14035 A2J9 and E14035 A2J12 showed a 2fold inhibition of NO production from 0.993 ± 0.012 μM to 0.572 ± 0.0019 μM and 0.572 ± 0.0012 μM, respectively. It seems that our subfractions had a lower strength of inhibition; however, the comparison is difficult, since the observed inhibition from our subfractions was nearly 100%, reaching the same level as the solvent control. Higher sensitivity could only be shown by increasing the induction of NO production by LPS or by testing the pure, responsible compound of the mixture from the subfraction.

By the interpretation of the NMR' spectra of these fractions, we can infer that they are not pure compounds yet; it seems that some of the compounds are present in all these fractions, which could be analogues. More analyses have to be done to conclude, which compounds are present in the bioactive fractions. Another round of purification has to be done to isolate the compound, however the final weight that we have to perform this is not

compatible with the purification process. Therefore, it requires a new period to allow the growth of this strain, and after the isolation of the compound, 1D and 2D NMR can be used to perform the elucidation of the chemical structure. LC/MS of these subfractions will be performed in the near future.

Once a cyanobacterial compound is isolated and characterized, future studies will focus on the mechanistic pathway through which these compounds are able to exert their effects.

Comparing the levels of inhibition of NO production with previous studies that used the same methodology in RAW 264.7 cells (Yang *et.al.*, 2012), we can conclude that these last sub-fractions have some potentiality for anti-inflammatory compounds. As Yang *et.al.* (2012) showed before, 6,6′-bieckol – a compound isolated from a brown alga (*Ecklonia cava*) – significantly inhibited LPS-stimulated NO and PGE_2 production when treated with 100 and 200 μM in RAW 264.7 cells. This compound decreased, at these concentrations, the NO production from 50 μM to less than 20 μM, what proves that 6,6′-bieckol has anti-inflammatory activity. Comparing to the LPS induction treatment, the fractions with anti-inflammatory activity (A2J7, A2J9, A2J10 and A2J13) inhibited almost 50% the NO production to the same level of the solvent control. For that reason, we can suppose that it leads to an absence of inflammation in macrophage cell line.

Previous studies suggested that eicosapentaenoic acid (EPA), an Omega 3 Fatty Acid (ω-3 FA) was able to inhibit the NO production. The decrease in NO production occurred at the level of iNOS protein expression shown by western blot. The treatment with LPS at 1 μg/ml in RAW 264.7 cells revealed a NO production of 29.31 ± 0.57 μM after 24 hours, and this effect was reduced by pre-treatment with 1.44 $\mu l.ml^{-1}$of ω-3 FA (Corresponding to a concentration of 100 μM of EPA) to a 3.00 ± 0.99 μM NO (Aldrige *et.al.*, 2008).

As referred before, flavonoids and alkaloids have anti-inflammatory activity, so they could be constituents of the bioactive compounds in our case.

C-phycocyanin (C-PC), as already mentioned before, is a major component with interesting bioactivities from cyanobacteria. C-PC's that come from different species of cyanobacteria, such as *Aphanizomenon* sp.*, Spirulina* sp.*, Phormidium* sp.*, Lyngbya* sp.*, Synechocystis* sp.*, Synechococcus* sp. have already been isolated and investigated. C-PC is an anti-inflammatory protein, since it inhibited NO production and iNOS expression in RAW 264.7 cells, probably due to the suppression of TNF-α synthesis and nuclear NF-kB activation (Rahman, 2008).

More investigation and efforts should be applied in the future in the case of the strain LEGE 03283. It would be essential to perform a large scale production of biomass from

this strain in order to isolate the bioactive compound, to determine the IC_{50} and to assess the cytotoxicity of the compound. Furthermore, other techniques could be applied to characterize its inflammation potential, such as, analysis of mechanisms *in vitro* that have different targets as the cytokines TNF-α or IL-6. Villa *et.al*. (2010 (b)) used in their research ELISA and quantitative real-time Polymerase Chain Reaction (qRT-PCR) to analyze cytokines and enzymes involved in the inflammation process. Some inflammation reliefs are known to inhibit the COX enzymes (Koeberle & Werz, 2014). Strategies to evaluate these enzymes and cytokines involved in the inflammation process would be an interesting way to continue the work of cyanobacterial compounds with anti-inflammatory activities. Also, it needs to be evaluated if the compound in study has multiple mechanisms of action or just one.

Moreover, we could perform a study *in vivo*, for example. After the isolation of the compound with strong anti-inflammatory potential, it should be tested in a more complex model system, like Wistar rats. Alolga *et.al.* (2015) used Wistar rats in a previous study to evaluate the ability of Kang 601 heji (K-601), an ordinary Chinese herb used to treat cold and fever, to inhibit the inflammation process. For future works, we could use the same endpoints as in this previous investigation, in order to better understand the composition and the mechanisms of action behind the cyanobacteria *Aphanzomenon* sp. LEGE 03283.

Kang 601 heji (K-601) is a traditional Chinese preparation with therapeutically effects used for common cold comprising five herbs, *Lonicera japonica Thunb.* (L. japonica), *Isatis indigotica Fort*. (*I. indigotica*), *Rheum palmatum L.*, *Phellodendron chinense Schneid*. (*P. chinense*), and *Scutellaria baicalensis Georgi* (*S. baicalensis*). Chromatography analysis and mass spectra were executed to discover the constituents of the formulation K-601, and the results revealed it contains several compounds ranging from flavonoids, alkaloids, isoflavonoids, etc. The full outcome revealed 50 identified compounds. In the assays performed in this work, K-601 possesses the ability to reduce fever in a dose-dependent manner. The compound studied revealed an ability to reduce NO production, as measured by the Griess' method; however, the results in "NO concentration" were not available on the published paper. The possible mechanisms of actions of this formulation could be the inhibition of NO production by the cells, reduction of release of cytokines and the inhibition of PGE_2 release during inflammation. Flavonoids, that are included in its formulation, have been found to have anti-oxidative and free radical scavenging activities. These compounds are able to control some cellular processes of inflammation related cells, as, mast cells, macrophages, lymphocytes and neutrophils. Some of them modulate the enzyme activities of arachidonic acid, metabolizing enzymes such as cyclooxygenase, lipoxygenase, phospholipase A2, NO synthase. In that way, the inhibition of these

enzymes leads to a decrease in arachidonic acid, leukotrienes, prostaglandins and NO, which are crucial mediators of the inflammation process. The anti-inflammatory ability of flavonoids could be related to the inhibition of the nuclear factor kappa B transcription activation (Alolga *et.al.*, 2015). The alkaloids constituents have demonstrated to be strong anti-inflammatory agents, as for example, the alkaloidal fraction from *Alstonia scholaris* including picrinine, vallesamine and scholaricine that showed strong anti-inflammatory activity. The IC_{50} values suggested that compounds 5–14, all indole aglycones, exhibited potent inhibitory activities against NO production, with IC_{50} values of 5.34 to 13.45 μM (the positive control, indomethacin, had an IC_{50} value of 14.10 μM). These results cannot be directly compared with ours since have a mixture of various compounds and do not have the chemical structure of the responsible compounds. However, the chosen concentration of 30 μg/ml in our screening assays reveals that we have observed relevant anti-inflammatory activities in the cyanobacterial fractions. In comparison, the IC_{50} concentration of the model compound indomethacin (14 μM) corresponds to approximately 5 μg/ml, but is already a solution of a pure and single compound.

Bioactivity guided-fractionation vs other methodologies

Bioactivity guided-fractionation is a common method used to discover novel natural compounds that focusses on extract preparation, fractionation, biological screening with appropriate assays and further, the process of isolation, purification and structural elucidation of the active compounds (Oldoni *et.al.*, 2016; Bargougui *et.al.*, 2014; Ding *et.al.*, 2013). This technique has been very helpful for the discovery of compounds for specific targets, and generally appeals to chromatographic techniques as liquid chromatography, HPLC, liquid-liquid or solid phase extraction (Oldoni *et.al.*, 2016). However, bioactivity guided fractionation has the disadvantage of being time consuming and expensive, since high volumes of organic solvents are used, and sometimes these solvents are hardly compatible with bio- or biochemical assays (Cutignano *et.al.*, 2015; Weller, 2014). Moreover, this process has the disadvantage of the possibility to elucidate already described compounds from the literature (Katiyar *et.al.*, 2012). Synergisms and antagonisms between compounds can occur that can ultimately result in the loss of activity or the blocking of the activity of a compound, respectively (Koeberle & Werz, 2014). Another limitation is that the method is resource consuming. Each fractionation that is executed for a compound requires a preparation of new exposure solutions, which results in losses of biomass of the sub-fractions (Yuliana *et.al.*, 2013). If the bioassay fails, additional biomass is needed to prepare new solutions and to re-test, as happened in

some of the fractions in this present work. However, this approach ensures that a compound with a specific biological activity is identified. Some investigations followed this methodology with interesting outcomes, as the case of the discovery of proanthocyanidins with anti-oxidant activity (Oldoni *et.al.*, 2016), polyphenols with anti-tumor activity from the extract of *Rhus verniciflua* (Kim *et.al.*, 2013), hierridin B, extracted from the marine picocyanobacterium *Cyanobium* sp. LEGE 06113 with anti-tumor activity (Leão *et.al.*,2013), or lignans from the root of *Machilus obovatifolia* (Lin *et.al.*, 2015), between others.

In contrast, different approaches exist for the isolation of novel secondary metabolites. Basically, these approaches make the opposite of the previous one. It is based on the identification and elucidation of the structure of new compounds, before the activity will be analyzed in bioactivity assays. Isolated compounds with chemical identities of interest are screened for specific bioactivities, a process that contrasts with the classic bioactivity guided-fractionation. This methodology allowed the discovery and isolation of a variety of bioactive compounds, such as the xanthones from the species *Garcinia succifolia* (Duangsrisai *et.al.*, 2014). The disadvantage of this approach is that sometimes novel compounds are described that does not have any relevant bioactivity.

The process of dereplication technique is often used in an early stage of the work, before the isolation and characterization of compounds in order to discriminate already characterized compounds. The crude extract is analyzed by spectroscopy and supplementary analyses are done just for the compounds with non-characterized features, which allow a higher probability to elucidate and identify novel secondary metabolites. The most common methodologies are UV spectroscopy and mass spectrometry combined with HPLC (LC-UV/PDA, LC-MS) that allow the identification of certain structural classes and gives leads on the molecular weight and formula (Hubert *et.al.*, 2014).

However, they often lack in flexibility and a previous method development is needed, which consumes time and money. Therefore, from this point of view, a bioactivity guided fractionation is a more versatile technique. In our lab, this approach allows to find novel secondary metabolites with a larger range of bioactivities and to explore the cyanobacterial biotechnological and pharmaceutical potential.

6. Conclusion

Through the years, it is recognized that pathologies associated with inflammation are a huge health problem. People rely on medicines to control the pain associated to the inflammation processes, which have several side effects, like NSAID's. This work aimed to identify a novel compound with anti-inflammatory activity from a natural resource, preferably with less adverse effects. Cyanobacteria are known for the ability to produce bioactive compounds, some with potential application in this area. This is particular true for marine cyanobacteria, since freshwater cyanobacteria species such as *Aphanizomenon flos-aquae*, *Spirulina platensis*, *Spirulina maxima* and *Nostoc commune* have been used for feed and cosmetics (Raja *et.al.*, 2016). Although different bioactivities of cyanobacteria are reported, especially in what concerns to human health and well-being, just few information are available on the potential uses of these organisms for anti-inflammatory treatment. 117 fractions from 13 cyanobacterial strains derived from the cyanobacterial culture collection of CIIMAR (BEE group) were screened for their first time for their anti-inflammatory potential.

A fraction from *Synechocystis* sp. LEGE07211 (E14031D), a more simple cyanobacteria belonging to the Chroccocales genus, showed strong anti-inflammatory activity in RAW 264.7 cell line. These results confirm that unicellular free-living species of cyanobacteria are prolific in secondary metabolites with bioactivity of interest.

The strain *Phormidium sp.* LEGE 06363 with the sub-fraction E14026 A7 revealed strong potential for anti-inflammatory activity; however it was not possible to isolate the responsible compound, since the available biomass was not sufficient for a complete isolation.

The fractions E14035 A2J9 and E14035 A2J12 from *Aphanizomenon* sp. LEGE 03283 from the order *Nostocales* revealed strong anti-inflammatory activities. Again, the biomass of the sub-fractions was not sufficient to complete the isolation procedure, and final NMR spectra indicated the presence of more than one compound.

Even though this research supplies data about the potential properties of the strains, further research efforts must be conducted. Further biomass productions have to be done in the future to provide the raw material for successful isolations. Obtained results can be used to start with efficient chromatographic procedures to isolate and purify the responsible compounds of the anti-inflammatory activity. A new and different methodology should be applied to assess the anti-inflammatory activity in order to complement the data developed in this work. For example, zebrafish embryos offer a fast and sensitive

methodology to screen for anti-inflammatory activities, which delivers on the same time information about potential toxic side effects *in vivo*.

To the best of our knowledge, this is the first work focusing on bioactivity guided isolation of novel cyanobacterial secondary metabolites for anti-inflammatory pathologies or diseases using the cyanobacterial culture collection of CIIMAR.

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- www.jax.org
- www.biospectrum.com
- http://www.ciimar.up.pt/legecc/

Appendixes

Appendix A - ^{1}H NMR data for sub-fractions resulting from E14035A2 successive fractionings.

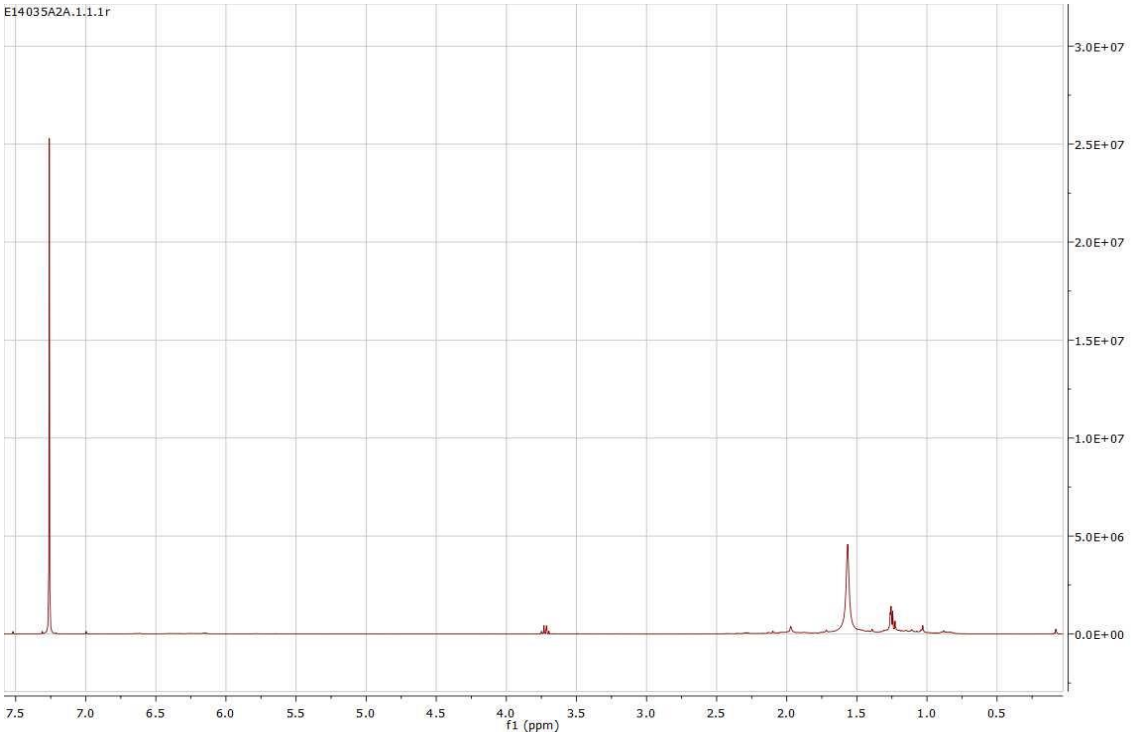

Figure 1 – ^{1}H NMR spectral data for E14035A2A in $CDCL_3$ (recorded at 400 MHz).

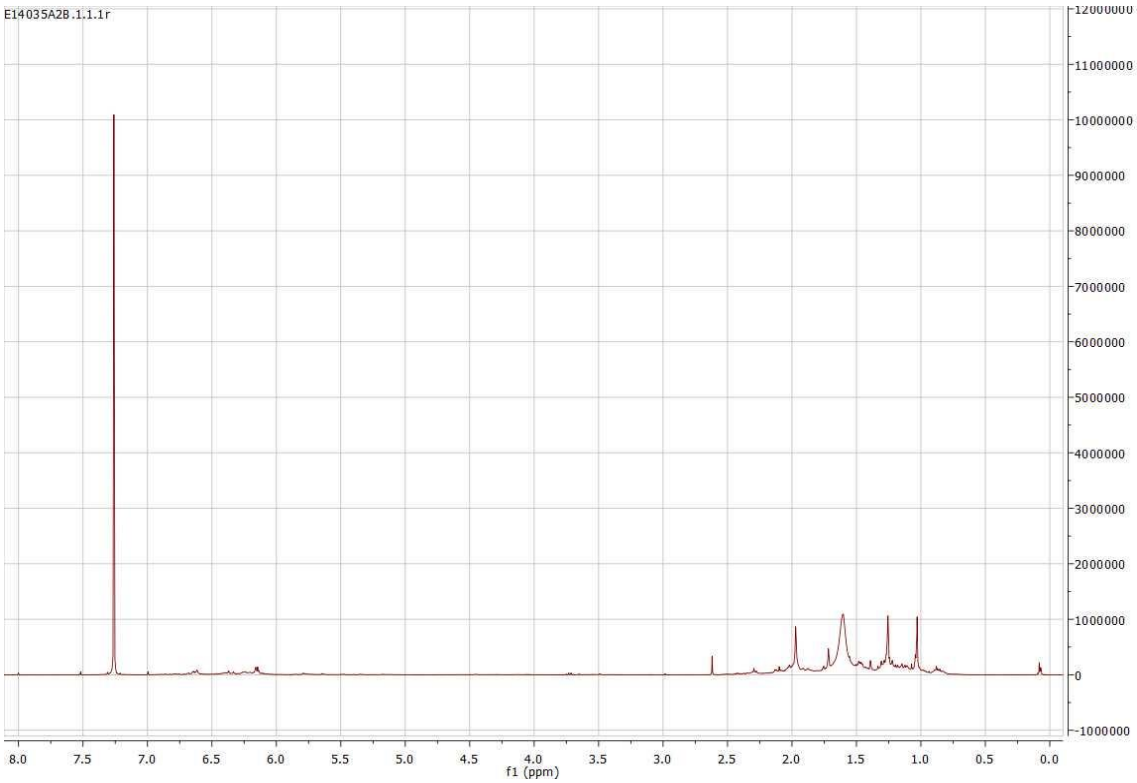

Figure 2 – ^{1}H NMR spectral data for E14035A2B in $CDCL_3$ (recorded at 400 MHz).

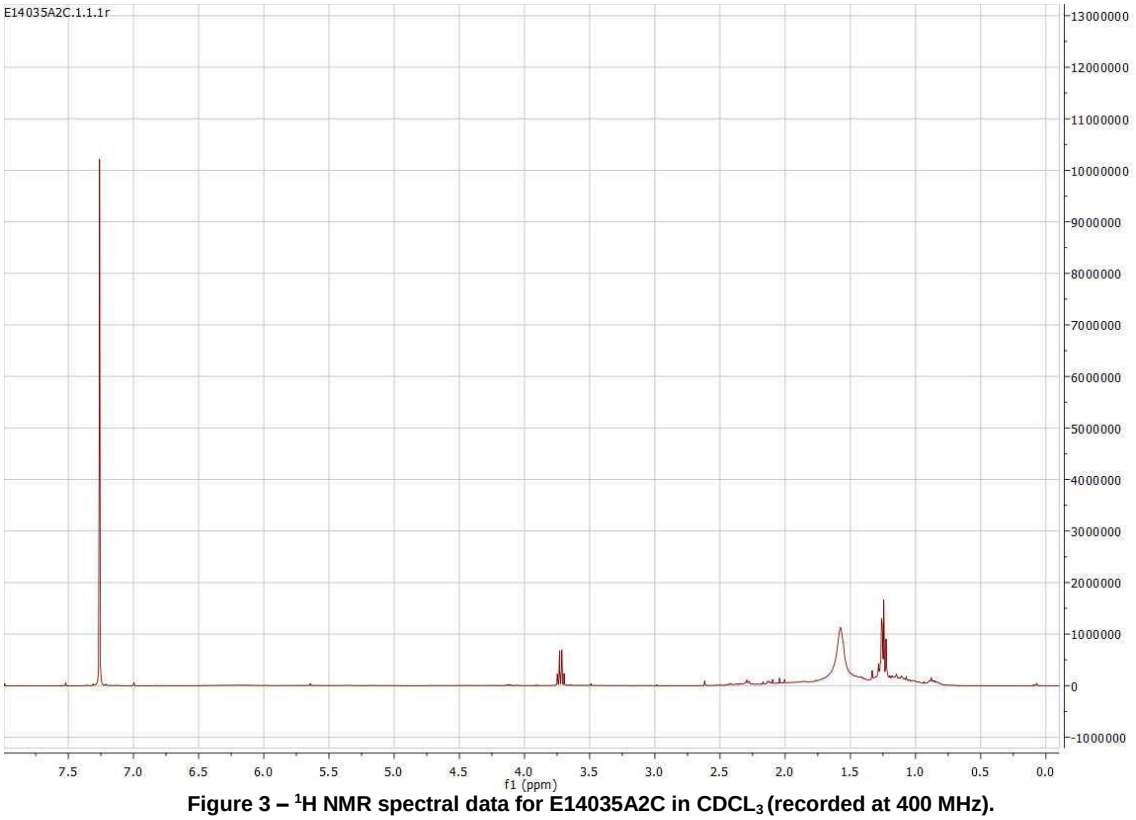

Figure 3 – ^{1}H NMR spectral data for E14035A2C in $CDCL_3$ (recorded at 400 MHz).

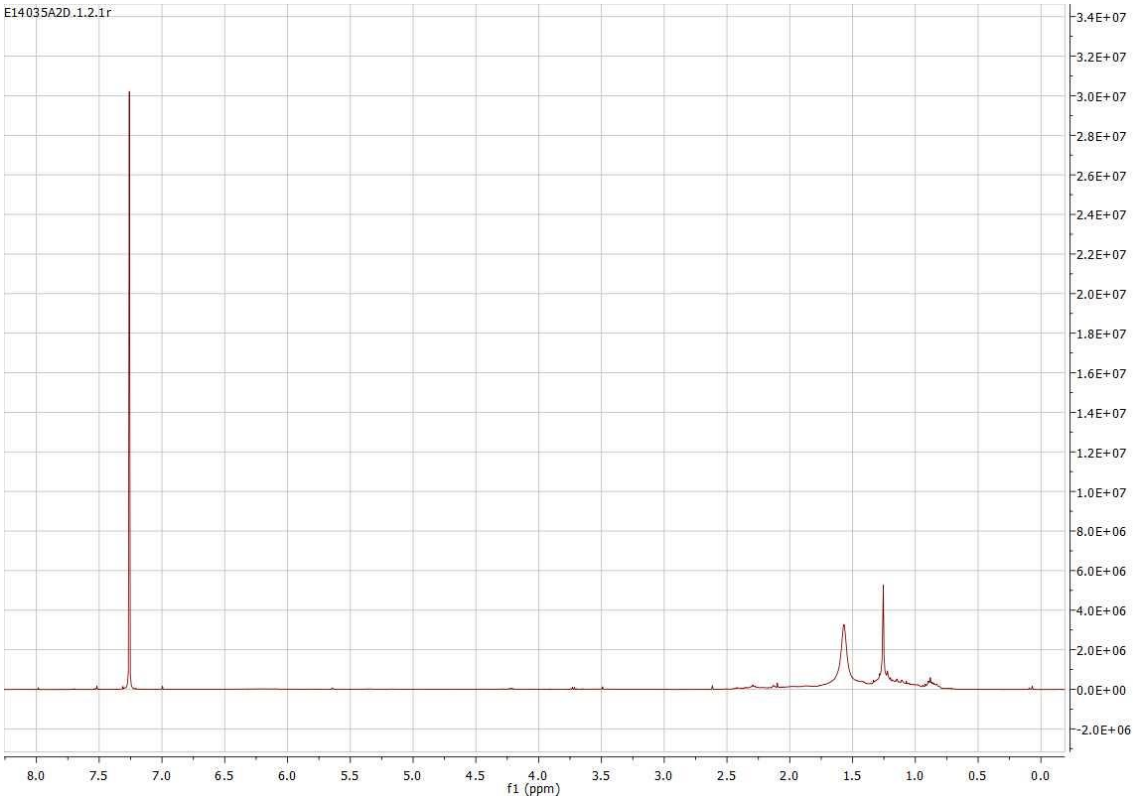

Figure 4 – ^{1}H NMR spectral data for E14035A2D in $CDCL_3$ (recorded at 400 MHz).

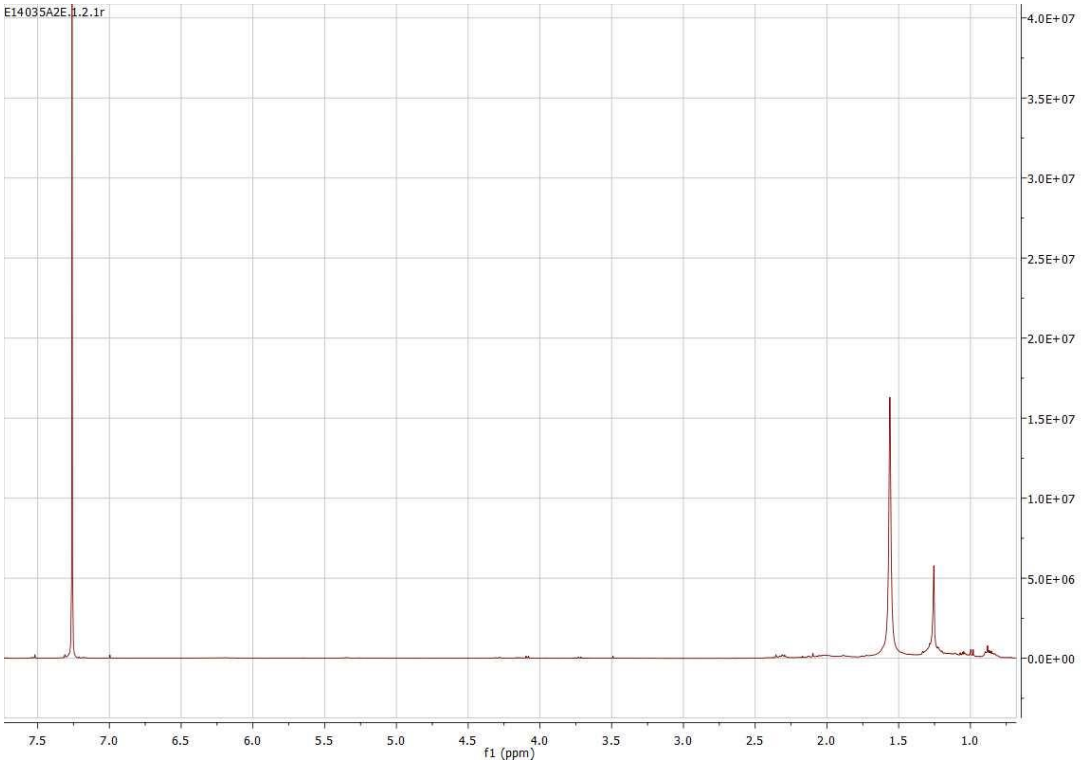

Figure 5 – ^{1}H NMR spectral data for E14035A2E in $CDCL_3$ (recorded at 400 MHz).

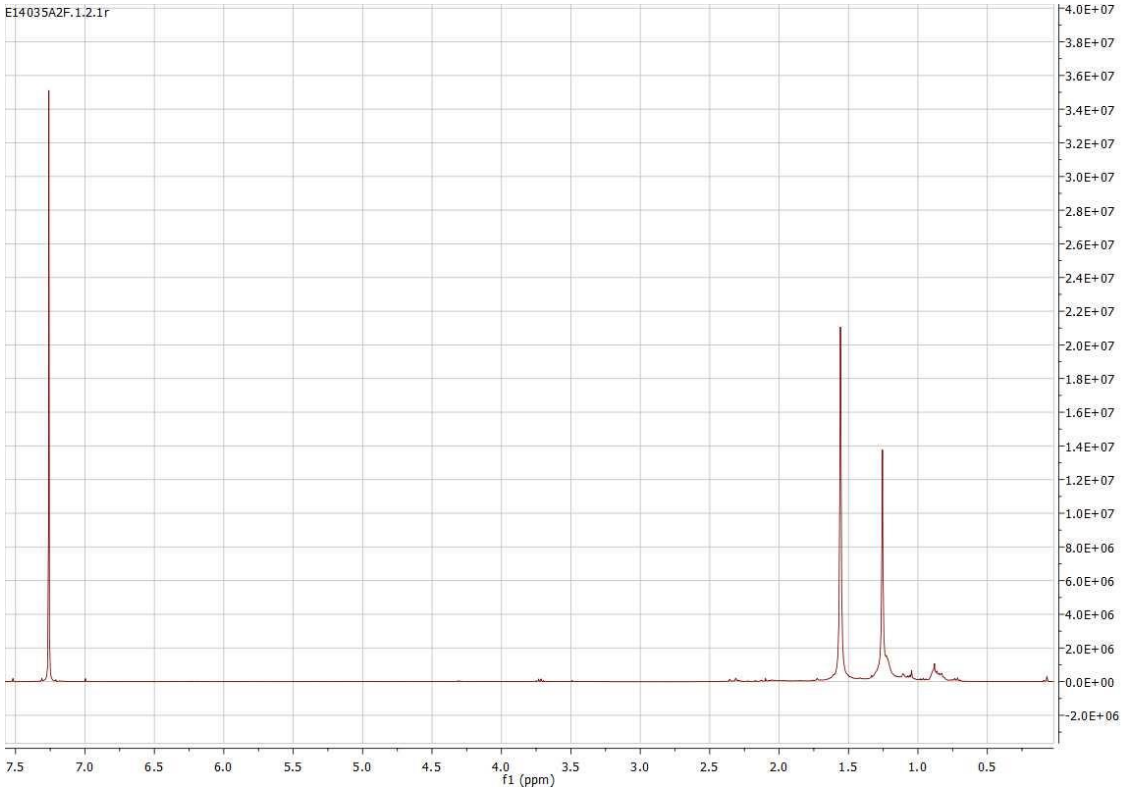

Figure 6 – ^{1}H NMR spectral data for E14035A2F in $CDCL_3$ (recorded at 400 MHz).

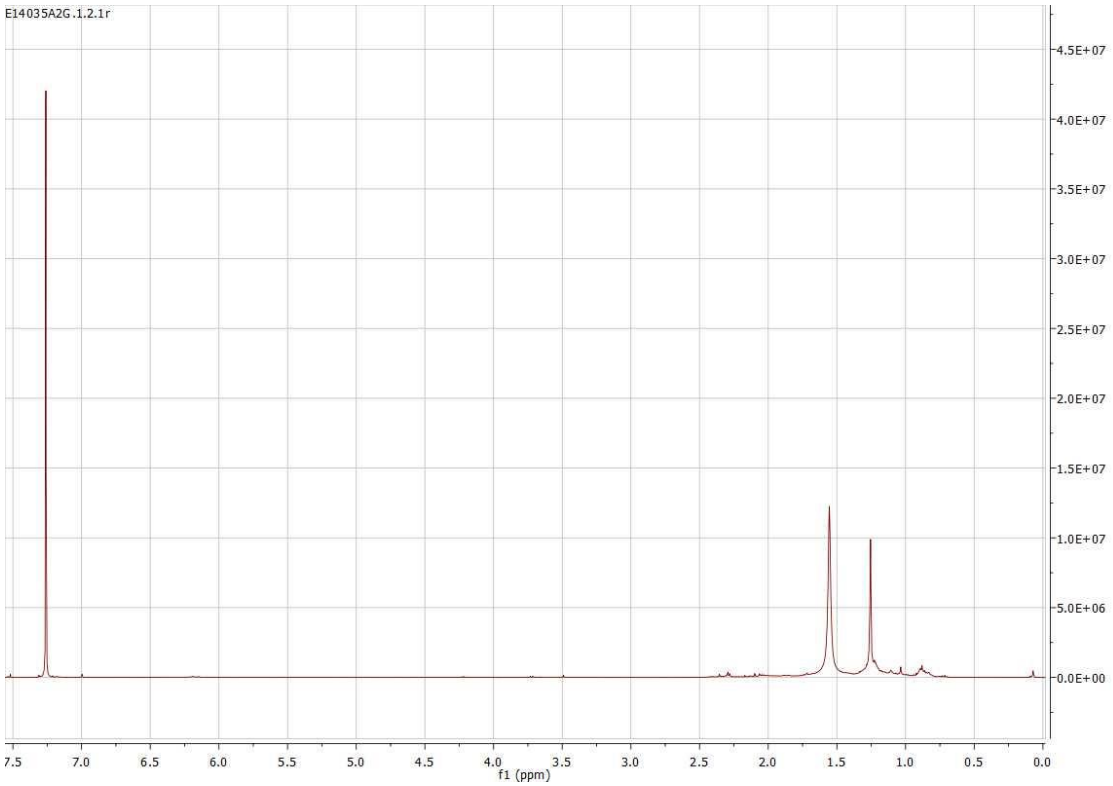

Figure 7 – ^{1}H NMR spectral data for E14035A2G in $CDCL_3$ (recorded at 400 MHz).

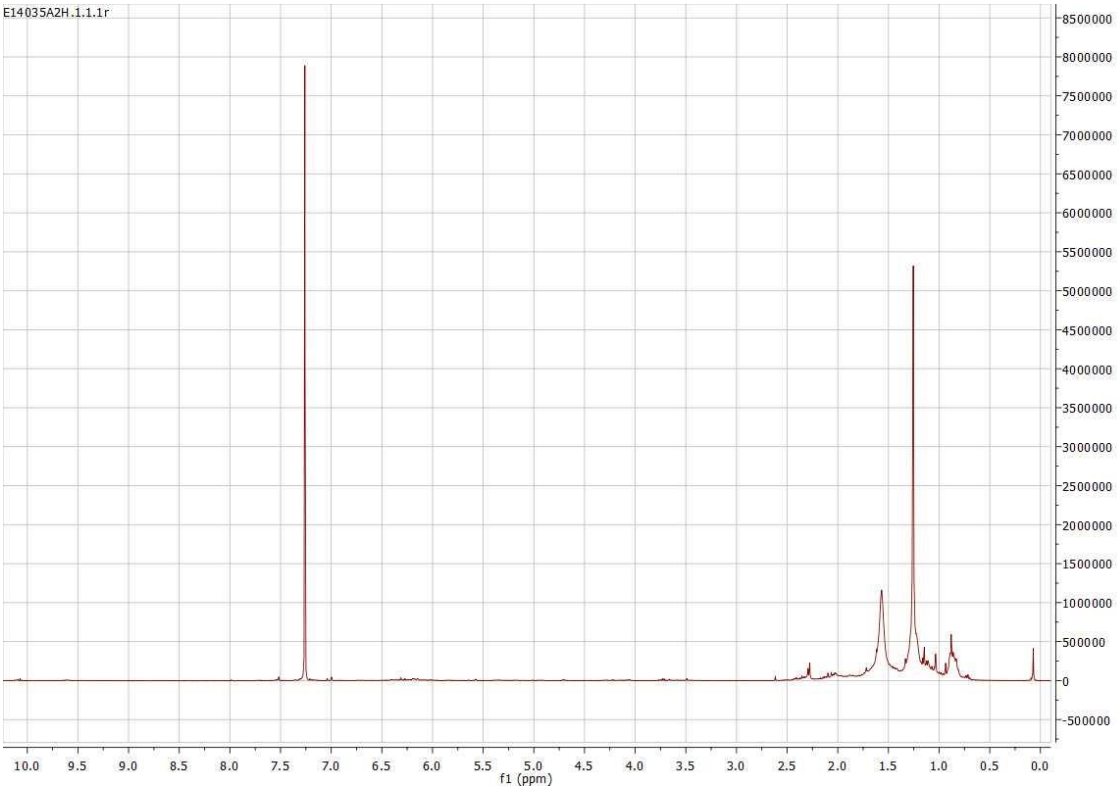

Figure 8 – ^{1}H NMR spectral data for E14035A2H in $CDCL_3$ (recorded at 400 MHz).

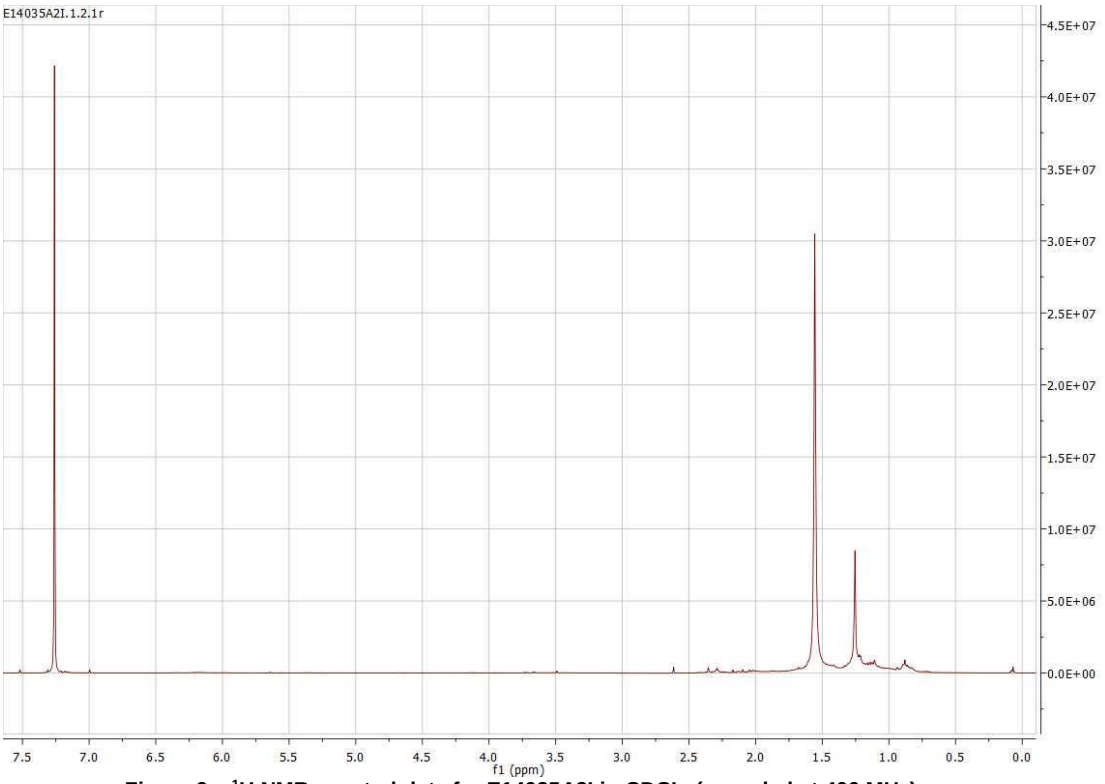

Figure 9 – ^{1}H NMR spectral data for E14035A2I in $CDCL_3$ (recorded at 400 MHz).

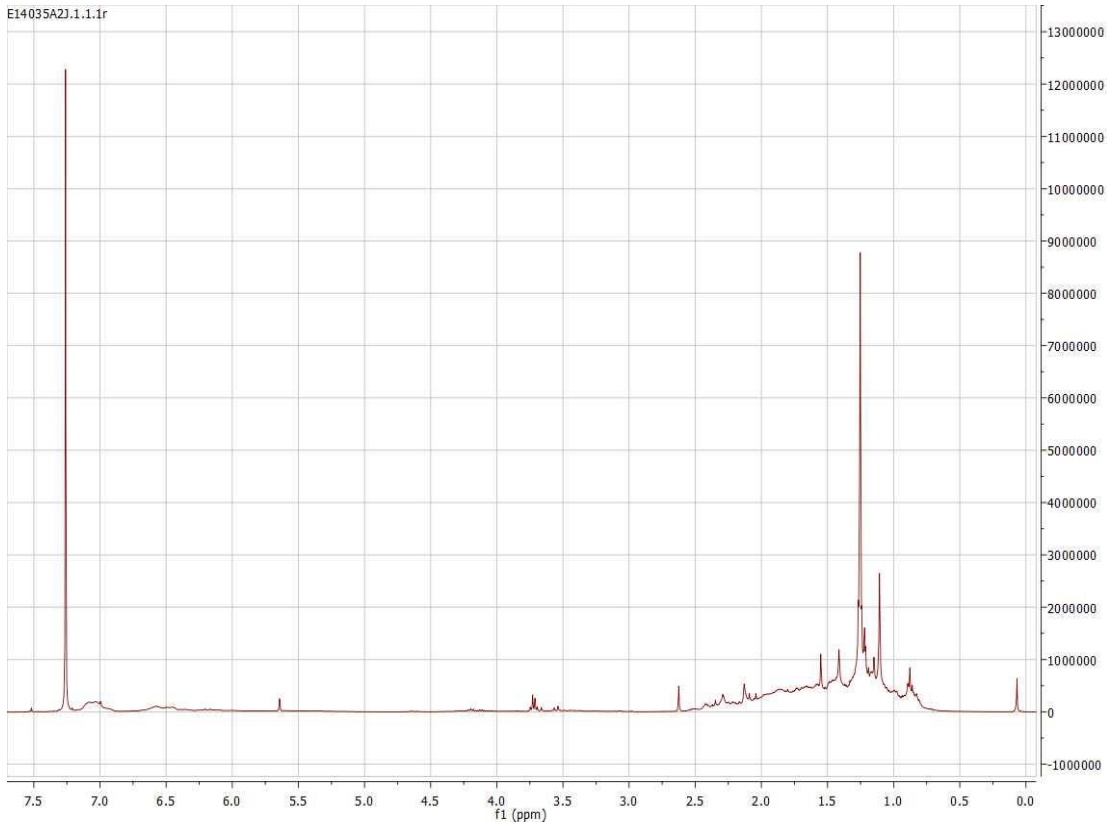

Figure 10 – ^{1}H NMR spectral data for E14035A2J in $CDCL_3$ (recorded at 400 MHz).

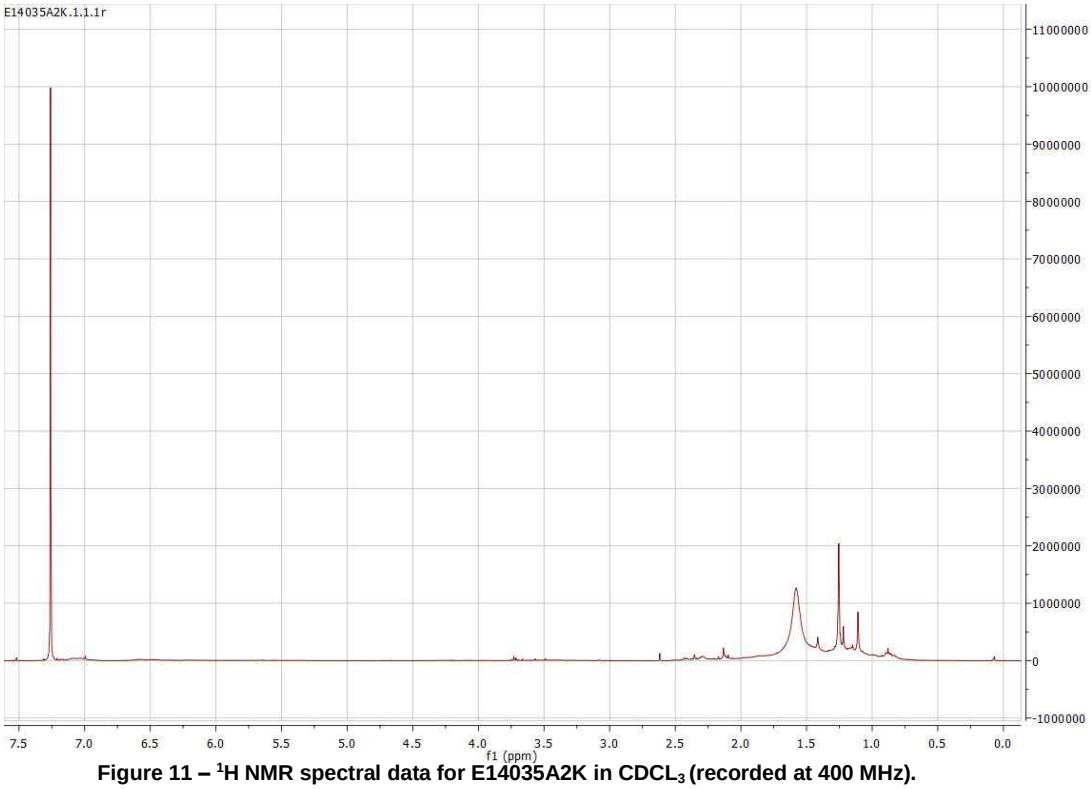

Figure 11 – ^{1}H NMR spectral data for E14035A2K in $CDCL_3$ (recorded at 400 MHz).